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MOLECULAR GENETIC STUDIES OF MELANOCORTIN RECEPTORS IN MORBID OBESITY

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original articles, which are referred to in the text by the Roman numerals I–IV. In addition, some unpublished data are presented.

I. Schalin-Jäntti C, Valli-Jaakola K, Oksanen L, Martelin E, Laitinen K, Krusius T, Mustajoki P, Heikinheimo M, Kontula K: Melanocortin-3-receptor gene variants in morbid obesity. *Int J Obes Relat Metab Disord.* 2003; 27:70-4.

II. Valli-Jaakola K, Lipsanen-Nyman M, Oksanen L, Hollenberg AN, Kontula K, Bjorbaek C, Schalin-Jäntti C. Identification and characterization of Melanocortin-4-Receptor Gene Mutations in Morbidly Obese Finnish Children and Adults. *J Clin Endocrinol Metab.* 2004; 89:940-5.

III. Valli-Jaakola K, Palvimo JJ, Lipsanen-Nyman M, Salomaa V, Peltonen L, Kontula K, Schalin-Jäntti C. A Two-Base Deletion -439delGC in the Melanocortin-4 Receptor Promoter Associated with Early-Onset Obesity. *Horm Res.* 2006; 66:61-69.

IV. Valli-Jaakola K, Suviolahti E, Schalin-Jäntti C, Ripatti S, Silader K, Oksanen L, Salomaa V, Peltonen L, Kontula K. Association of an *ENPP1* haplotype with morbid obesity in Finnish adults. Submitted.

ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
AGRP	agouti related protein
ATCC	American Type Culture Collection
BDNF	brain-derived neurotrophic factor
bHLH	basic helix-loop-helix
BMI	body mass index
cAMP	cyclic adenosine monophosphate
CART	cocaine-and-amphetamine-related transcript
dHPLC	denaturing high performance liquid chromatography
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ENPP1	ectonucleotide pyrophosphatase phosphodiesterase 1
JAK	Janus kinase
LD	linkage disequilibrium
LEPR	leptin receptor
MC1R-MC5R	melanocortin receptor family member 1, 2, 3, 4 or 5
MSH	melanocyte stimulating hormone α-, β- or γ_1-
NHLH2	nescient helix-loop-helix 2
NPY	neuropeptide Y
OGTT	oral glucose tolerance test
PBS	phosphate buffered saline
PC1, PC2	prohormone convertase 1 or 2
PCR	polymerase chain reaction
POMC	pro-opiomelanocortin
PVN	paraventricular nucleus
QTL	quantitative trait locus
SDS	standard deviation score
SIM1	homolog of *Drosophila* single-minded 1
SNP	single nucleotide polymorphism
STAT	signal transducer and activator of transcription
TRH	thyrotropin releasing hormone
TrkB	tropomyosin-related kinase B
UTR	untranslated region
WHO	World Health Organization
WHR	waist-to-hip ratio
wt	wild-type

ABSTRACT

The prevalence of obesity is increasing at an alarming rate in all age groups worldwide. Obesity is a serious health problem due to increased risk of morbidity and mortality. Although environmental factors play a major role in the development of obesity, the identification of rare monogenic defects in human genes have confirmed that obesity has a strong genetic component. Mutations have been identified in genes encoding proteins of the leptin-melanocortin signaling system, which has an important role in the regulation of appetite and energy balance.

The present study aimed at identifying mutations and genetic variations in the melanocortin receptors 2-5 and other genes active on the same signaling pathway accounting for severe early-onset obesity in children and morbid obesity in adults.

The main achievement of this thesis was the identification of melanocortin-4 receptor *(MC4R)* mutations in Finnish patients. Six pathogenic *MC4R* mutations (308delT, P299H, two S127L and two -439delGC mutations) were identified, corresponding to a prevalence of 3% in severe early-onset obesity. No obesity causing *MC4R* mutations were found among patients with adult-onset morbid obesity. The *MC4R* 308delT deletion is predicted to result in a grossly truncated nonfunctional receptor of only 107 amino acids. The C-terminal residues, which are important in MC4R cell surface targeting, are totally absent from the mutant 308delT receptor. *In vitro* functional studies supported a pathogenic role for the S127L mutation since agonist induced signaling of the receptor was impaired. Cell membrane localization of the S127L receptor did not differ from that of the wild-type receptor, confirming that impaired function of the S127L receptor was due to reduced signaling properties. The P299H mutation leads to intracellular retention of the receptor. The -439delGC deletion is situated at a potential nescient helix-loop-helix 2 (NHLH2) -binding site in the *MC4R* promoter. It was demonstrated that the transcription factor NHLH2 binds to the consensus sequence at the -439delGC site *in vitro*, possibly resulting in altered promoter activity.

Several genetic variants were identified in the melanocortin-3 receptor *(MC3R)* and pro-opiomelanocortin *(POMC)* genes. These polymorphisms do not explain morbid obesity, but the results indicate that some of these genetic variations may be modifying factors in obesity, resulting in subtle changes in obesity-related traits. A risk haplotype for obesity was identified in the ectonucleotide pyrophosphatase phosphodiesterase 1 *(ENPP1)* gene through a candidate gene single nucleotide polymorphism (SNP) genotyping approach. An *ENPP1* haplotype, composed of SNPs rs1800949 and rs943003, was shown to be significantly associated with morbid obesity in adults. Accordingly, the *MC3R*, *POMC* and *ENPP1* genes represent examples of susceptibility genes in which genetic variants predispose to obesity.

In conclusion, pathogenic mutations in the *MC4R* gene were shown to account for 3% of cases with severe early-onset obesity in Finland. This is in line with results from other populations demonstrating that mutations in the *MC4R* gene underlie 1-6% of morbid obesity worldwide. *MC4R* deficiency thus represents the most common monogenic defect causing human obesity reported so far. The severity of the MC4-receptor defect appears to be associated with time of onset and the degree of obesity. Classification of MC4R mutations may provide a useful tool when predicting the outcome of the disease. In addition, several other genetic variants conferring susceptibility to obesity were detected in the *MC3R*, *MC4R*, *POMC* and *ENPP1* genes.

INTRODUCTION

The prevalence of obesity is increasing at an alarming rate in all age groups worldwide (Kopelman 2000; Hill 2006). Although this trend is largely attributable to environmental changes, such as decreased physical activity and easy access to high-energy containing food, adoption and twin studies have clearly demonstrated that obesity has a strong genetic component (Bell et al. 2005).

During the last ten years there has been remarkable progress in the field of genetics. The recent advances in genetic research technologies, the completion of the Human Genome Project (International Human Genome Sequencing Consortium 2004) and the increasing amount of genetic information publicly available in databases have provided the basic tools for the identification of disease-related genes. The cloning and characterization of several mouse obesity genes constitute the basis for current molecular genetic obesity research and have greatly increased the understanding of the regulation of appetite and energy balance (Robinson et al. 2000). The identification of rare monogenic defects in the equivalent human genes, encoding proteins of the leptin-melanocortin signaling system, has confirmed that obesity genes indeed exist. Functional testing of mutated proteins has become a prerequisite research tool in the evaluation of the consequences of a genetic defect. The knowledge about conserved non-coding sequences is increasing and it is becoming obvious that regulatory regions containing binding sites for transcription factors are important in the regulation of gene expression.

Although several genes involved in the regulation of energy balance and appetite have been identified, the genetic basis of common obesity remains partly unsolved. Over 250 chromosomal loci linked with obesity-related phenotypes and 127 different candidate genes associated with obesity have been identified (Rankinen et al. 2006). The next goal in the post-genome era is to further characterize the variation in the human genome and to identify the factors underlying common, polygenic forms of obesity. The integration of various techniques and information from several sources, e.g. environmental factors, genotype information and gene expression data, will provide the means to clarify this field.

The main purpose of the present work was to investigate if severe early-onset obesity in children and morbid obesity in adults in Finland can be explained by genetic variation in the melanocortin receptors 2-5 and other genes active in the same signaling pathway.

REVIEW OF THE LITERATURE

1 OBESITY

1.1 Definition of obesity

Obesity can be defined as an excess of fat tissue resulting from a long term imbalance between energy intake and expenditure (WHO 1995; WHO 2000). A simple index that is commonly used in adults to classify obesity is the body mass index (BMI), which is calculated by dividing the weight in kilograms by the square of the height in meters (kg/m^2). The cut-off points proposed by the World Health Organization (WHO) in the classification of obesity are shown in Table 1 (WHO 1995; WHO 2000). The classification is based primarily on the association between BMI and mortality (Manson et al. 1995; WHO 1995; WHO 2000)

Table 1. Cut-off points in the classification of obesity in adults proposed by the WHO (WHO 1995; WHO 2000).

BMI (kg/m^2)	WHO classification	Risk of obesity-related comorbidities
<18.5	Underweight	Low
18.5-24.9	Normal weight	Average
25.0-29.9	Overweight	Increased
30.0-34.9	Obese class I (Obesity)	Moderate
35.0-39.9	Obese class II (Severe obesity)	Severe
≥40.0	Obese class III (Morbid obesity)	Very severe

The classification of obesity status in children and adolescents is complicated because their height and body composition is continuously changing. BMI cut-off points for defining overweight and obesity in children have been proposed by Cole et al. (2000), but no widely accepted classification criteria for childhood obesity are available at present. In Finland the recommendation is to use relative weight for height and compare it to age specific 90. and 98. percentile curves (Table 2) (Sorva et al. 1984). The adult BMI cut-off points are applicable when growth in height has ended.

Table 2. Cut-off points used in the classification of childhood obesity in Finland.

Classification of obesity	Children of age under 7-years (weight for height, %)	Children from the age of 7-years - end of growth in height (weight for height, %)
Overweight	10-20	20-40
Obesity	> 20	> 40

Obese individuals with abdominal fat distribution are at high risk for obesity-associated illnesses (Kissebah and Krakower 1994). Abdominal fat accumulation can be measured anthropometrically by use of the waist-to-hip ratio (WHR) and waist circumference (Han et al. 1997; Janssen et al. 2004). More accurate measures to estimate body adiposity and fat distribution include e.g. underwater weighing, bioimpedance analysis, dual-energy X-ray absorptiometry, skinfold thickness, computer tomography, ultrasound and magnetic resonance imaging (WHO 2000).

1.2 Epidemiology

The prevalence of obesity and overweight is increasing worldwide at an alarming rate (Kopelman 2000; Hill 2006). The large increase in the prevalence of obesity in the United States has been documented by the National Health and Nutrition Surveys (Flegal and Troiano 2000). The number of obese individuals has doubled since the year 1980 in the US. Currently over 30% of the adult population can be considered obese (BMI > 30 kg/m^2) and 65% overweight (BMI > 25 kg/m^2). The prevalence of morbid obesity (BMI > 40 kg/m^2) has increased markedly in the US and is currently 4.7% among the adult population (Flegal and Troiano 2000). The most comprehensive data on obesity in Europe, collected between 1983 and 1986, comes from the MONICA study (Keil and Kuulasmaa 1989). At that time, more than half of the European population was overweight and 15% of men and 22% of women were obese. In Finland, the prevalence of obesity has increased between years 1982 and 1997 in men from 15.4% to 19.8% and in women from 17.7% to 19.4% (Lahti-Koski et al. 2000). Morbid obesity in Finland, with a prevalence of 0.5% in men and 1.2% in women, is rare if compared to the figures from the US (Pietinen et al. 1996). Increasing obesity rates are not problems of Europe and the US alone. The obesity epidemic is also affecting e.g. Southeast Asia (Popkin 1994), the Pacific region (Bennett and Magnus 1994; Hodge et al. 1995), Middle East (al-Nuaim et al. 1996) and even the African region (Steyn et al. 1991; Hodge et al. 1996).

Because of the lack of worldwide criteria for the classification of obesity in childhood and adolescence, there are no global estimates of the prevalence of obesity in younger age groups (WHO 2000). However, several national studies have reported that the prevalence of childhood obesity has increased (Freedman et al. 1997; Kotani et al. 1997; Ogden et al. 2002; Hedley et al. 2004). In the US the number of overweight and obese children has doubled during the last two decades (Freedman et al. 1997) and the same development is observed worldwide (Deckelbaum and Williams 2001). The trend is similar in Finland, the age-standardized prevalence of overweight among adolescents increased in boys from 7.2 to 16.7%, and in girls from 4.0 to 9.8% between years 1977 and 1999 (Kautiainen et al. 2002).

1.3 Morbidity and mortality associated with obesity

Obesity is associated with an increased risk of adverse health consequences (National Task Force on the Prevention and Treatment of Obesity 2000; Kopelman 2000). It is a burden to the public health care system and contributes to health care expenditures (Seidell 1995; Colditz 1999; Pekurinen et al. 2000; Anderson et al. 2005). Obesity predisposes to several diseases, e.g. type 2 diabetes, coronary heart disease, sleep apnea, stroke, gallbladder disease, liver disease, osteoarthritis, infertility and certain forms of cancer (National Task Force on the Prevention and Treatment of Obesity 2000; Kopelman 2000). Increased waist circumference is associated with increased health risk, even when comparing individuals with the same BMI (Janssen et al. 2004). Furthermore, abdominal fat distribution is a risk factor for obesity-associated illness and predisposes for hypertension, dyslipidemia, hyperinsulinemia and other metabolic disturbances (Kissebah and Krakower 1994).

Obese individuals are at high risk for developing insulin resistance and type 2 diabetes (Kahn et al. 2006). Insulin resistance can be defined as a decreased biological effect of insulin in target tissues (Kahn 1978), leading to an imbalance in glucose homeostasis. In the normal state, insulin stimulates the uptake and use of glucose by muscle and adipocytes and suppresses glucose production in the liver. This leads to lower

blood glucose levels. Several factors involved in the development of insulin resistance, e.g. free fatty acids, hormones and cytokines, are secreted by the adipose tissue. In obesity, increased amounts of these circulating factors together with impaired insulin secretion results in hyperglycemia and ultimately in type 2 diabetes (Kahn et al. 2006). The increasing prevalence of obesity is resulting in increased prevalence of the metabolic syndrome (Eckel et al. 2005), which can be defined as the co-occurrence of obesity-associated disturbances, e.g. central obesity, insulin resistance, hyperlipidemia and hypertension. Individuals with the metabolic syndrome are at high risk for developing type 2 diabetes and cardiovascular disease (Eckel et al. 2005).

Several studies have shown J- or U- shaped relationships between BMI and mortality, meaning that individuals from both ends of the curve are at high risk (Troiano et al. 1996). The mortality rate increases markedly with a BMI of at least 30 kg/m^2 (Troiano et al. 1996; Calle et al. 1999) and can be explained by chronic diseases that occur more frequently in obese than in lean individuals (Kopelman 2000).

The health effects associated with childhood obesity are similar to those observed in the adult population (Deckelbaum and Williams 2001). Hypertension, dyslipidemia and insulin resistance appear frequently in obese pediatric patients (Deckelbaum and Williams 2001). A particular problem is the increasing rates of type 2 diabetes associated with childhood obesity (Pinhas-Hamiel et al. 1996; Haines et al. 2007). Furthermore, obesity in childhood and adolescence is a key predictor of obesity in adulthood and increases the risk of obesity-associated diseases later in life (Goran 2001).

2 STRATEGIES TO STUDY THE GENETIC BACKGROUND OF OBESITY

2.1 Genetic epidemiology

Obesity is a complex trait, not solely following the rules of Mendelian recessive or dominant inheritance explained by the effect of a single gene. In complex traits, the interaction of both genetic and environmental factors predispose to the development of the disease (Lander and Schork 1994). The first evidence that genetics is involved in the development of common obesity came from genetic epidemiological twin studies, giving high estimates (> 0.78) for the heritability of obesity (Feinleib et al. 1977; Stunkard et al. 1986a). An adoption study performed by Stunkard et al. (1986b) gave similar estimates on the heritability of body weight. Clustering of obesity has been observed also in family studies (Heller et al. 1984; Bouchard et al. 1988). To date, a large number of epidemiological studies estimating the heritability of obesity have been published (for review, see Loos and Bouchard 2003). Different phenotypes, such as weight, BMI, WHR and skinfold thickness have been used in these studies. In general, these studies have given somewhat lower estimates for the heritability of obesity than the first twin and adoption studies. There is growing consensus that the heritability of obesity and body weight is about 30-40%, depending on the phenotype investigated (Bell et al. 2005).

Genetic epidemiology is an approach for studying the inheritance of a trait, based on statistical analysis of the distribution of phenotypes in related individuals (Bouchard 1995). In contrast, genotype approaches utilize information of the genetic variation on the level of deoxyribonucleic acid (DNA). The main strategies to study the genetic background of a human trait are genome-wide linkage, candidate gene and genome-wide association

approaches. Genome-wide association studies are increasingly utilized in successful gene identification.

2.2 Genome-wide linkage

When there is no pre-existing knowledge about the genes that might underlie a trait, a genome wide linkage approach can be used (Terwilliger and Ott 1994). The principle of a genome scan is to genotype polymorphic markers distributed over the whole genome. Traditionally microsatellite markers or other sequence repeats have been used. The degree of linkage between the markers and a disease trait is statistically calculated in families with several affected members. By use of linkage analysis it is possible to identify the chromosomal regions involved in disease susceptibility. If the chromosomal region is transmitted with the disease in families, it is likely that this region contains the gene involved in the disease pathogenesis. The method has been useful in identifying genetic regions and genes involved in the pathogenesis of monogenic diseases. However, there are also examples of identification of genes underlying complex diseases, such as *NOD2* in inflammatory bowel disease (Ogura et al. 2001) and *ADAM33* in asthma (Van Eerdewegh et al. 2002). In the field of obesity research, more than 250 quantitative trait loci (QTL) linked with obesity-related phenotypes have been identified (Rankinen et al. 2006). Of these QTLs identified, 52 were supported by evidence from two or more studies.

Several of these genome scans have studied linkage to BMI in families that were originally gathered for other purposes, e.g. sample sets for studies of type 2 diabetes, osteoporosis and hypertension (Bell et al. 2005). These cohorts may not be optimal for obesity research, as they have not been selected for body weight, BMI or other measures of adiposity. A sampling strategy useful in obesity research is to gather a sample set with a significant genetic component for obesity, by selecting families that include several extremely obese individuals (Bell et al. 2005).

2.3 Candidate gene approach

The candidate gene approach can be applied if there is some pre-existing knowledge about the disease studied (Tabor et al. 2002). There are two main types of candidate genes. Positional candidate genes are those that are identified through a genome scan, while functional candidates are genes that are thought to be involved in the pathogenesis of the disease. For example, studies with animal models, knowledge about metabolic pathways and gene expression profiling can provide information of potential functional candidates. A case-control approach represents the most common type of candidate gene study. Polymorphic markers are genotyped in the study population within the gene of interest, and tested for association with the disease. Another possibility is to resequence the gene in the study population in order to detect all variation within the gene. There are several examples of successful identification of obesity genes by use of the candidate gene approach (Rankinen et al. 2006). The latest human obesity gene map update lists 416 different reports of significant associations with 127 candidate genes (Rankinen et al. 2006). The first obesity genes that were identified by use of the positional candidate gene approach were *GAD2* (Boutin et al. 2003) and *SLC6A14* (Suviolahti et al. 2003a).

2.4 Rodent obesity loci and corresponding human genes

Animal models provide a valuable tool for studying the genetic background of complex traits (Lander and Schork 1994). The murine obesity gene map includes nearly 250 genes that, when mutated, knocked out or expressed as transgenes, result in phenotypes affecting body weight and adiposity (Rankinen et al. 2006). Studies with animal models may reveal homologous human genes or give information about other important genes that are involved in the same biological pathways (Lander and Schork 1994). The cloning and characterization of mouse *agouti, fat, tubby, obese* and *diabetes* genes have played a crucial role in obesity research and led to the identification of key neural circuits involved in the regulation of appetite and energy balance in mice and humans (Robinson et al. 2000). During the last years, several mouse QTLs have been fine mapped using rodent congenic strains (Rankinen et al. 2006). Several congenic rodent strains expressing a wide variety of adiposity and growth are available, providing a platform in which the analysis of QTLs is relatively easy. The advantage of studying polygenic obesity in animal models is that the environmental and genetic backgrounds can be held constant (Barsh et al. 2000). Additionally, mouse models have been developed in order to assess the question of interacting, epistatic loci that are suspected to play a major role in the genetics of complex traits (Warden et al. 2004).

2.5 New research strategies in the post-genome era

During the last ten years there has been a remarkable progress in the field of molecular genetics. The Human Genome Project provided the first draft of the human genome sequence in 2001 (Lander et al. 2001; Venter et al. 2001) and the remaining sequence gaps were finished in 2003 (International Human Genome Sequencing Consortium 2004). This sequence data, as well as information about genetic variation in the form of millions of single nucleotide polymorphisms (SNPs) (Sachidanandam et al. 2001), is available in public databases. The next goal in the post-genome era is to characterize the variation in the human genome and to identify the factors underlying complex diseases.

After the completion of the Human Genome Project, genomic sequences of several other organisms have also been released. Currently, the National Center for Biotechnology Information public database contains completed genomic sequences of 605 organisms and draft sequences of several other species. By comparing the genomes of different species, it has become apparent that the number of genes in the human genome does not differ markedly from the number of genes identified in other eukaryotes. The current estimate of the number of protein coding genes in human genome is around 20,000-25,000 (International Human Genome Sequencing Consortium 2004). There are also remarkable similarities in intronic and intragenic sequences between species. These conserved sequences are suspected to contain regions important in the regulation of gene expression, e.g. binding sites for transcription factors (Pennacchio and Rubin 2001). Information about transcription factor binding sites have been collected in databases, such as TRANSFAC (Wingender et al. 1996). This information can be used to search for binding sites for known transcription factors and for analysis of sequence variation detected in non-coding regions of the genome.

The International HapMap project started in 2002, with the goal of determining linkage disequilibrium (LD) patterns in the human genome in order to facilitate the research of complex diseases (The International HapMap Consortium 2003). Most of the recombinations in the human genome occur at same locations, in recombination hot-spots

(Gabriel et al. 2002). The SNPs located between the recombination hot-spots are in high LD with each other and constitute defined blocks in the genome. The variation within these blocks can easily be covered by selecting tag-SNPs. By use of these tag-SNPs it is possible to achieve maximal information about the genetic variation, with minimal genotyping efforts (The International HapMap Consortium 2003).

At the same time as a large amount of genetic information has become publicly available, there has also been enormous progress in SNP genotyping techniques (The International HapMap Consortium 2003). It is already possible to perform genome-wide association studies, which utilize the combination of association and linkage in gene identification. This approach has successfully identified loci for age-related macular degeneration (Klein et al. 2005), type 2 diabetes (Saxena et al. 2007), Alzheimer's disease (Coon et al. 2007), nicotine dependence (Uhl et al. 2007) and Crohn's disease (Libioulle et al. 2007; Rioux et al. 2007).

The recent development of microarray technology has provided a tool for gathering information on the simultaneous expression of thousands of genes in the tissue of interest (Lockhart and Winzeler 2000). In obesity research, this technique can be used to compare expression profiles from obese and lean individuals and to identify genes that are up- or downregulated in these physiological states (Bell et al. 2005). Large scale gene expression profiling and usage of expression data as QTLs has successfully been carried out in mice and humans (Schadt et al. 2003; Morley et al. 2004).

3 MONOGENIC ANIMAL MODELS OF SEVERE OBESITY

The cloning and characterization of several mouse obesity genes have played a crucial role in the field of obesity research and led to the identification of key neural circuits involved in the regulation of appetite and energy balance in mouse and human (Robinson et al. 2000). Some of the most important obesity mouse models, involved in melanocortin signaling or otherwise relevant for this thesis, are described in the following paragraphs.

3.1 Leptin (*ob/ob*) and the leptin receptor (*db/db*)

The obese *(ob/ob)* mouse strain presenting with autosomal recessive morbid obesity was reported in 1950 by Ingalls et al. The diabetes *(db/db)* mutation, discovered later, results in an almost identical phenotype as the *ob/ob* mutation (Coleman 1978). In the 1970's, parabiosis experiments were performed with these obese mouse strains. In these experiments the blood circulatory systems of *ob/ob* and *db/db* mice were connected with those of lean mice (Coleman 1973). The experiments suggested that the *ob/ob* mice lacked an adipostatic hormone and the *db/db* mice the receptor for that hormone (Coleman 1973). These results were confirmed in the 1990s when the *ob* and *db* genes were identified through positional cloning (Zhang et al. 1994; Tartaglia et al. 1995).

The *ob* gene encodes a protein belonging to the cytokine family, named leptin, which is secreted mainly from adipocytes (Zhang et al. 1994). The *db* gene encodes a receptor belonging to the family of class I cytokine receptors, which bind leptin with high affinity (Tartaglia et al. 1995). There are at least six alternatively spliced variants of the leptin receptor (LEPR), which vary in the length of their cytoplasmic domains (Lee et al. 1996). The long form of LEPR is expressed in hypothalamic regions of brain that are involved in the regulation of energy balance (Mercer et al. 1996). Furthermore,

homodimers of the long form are able to activate the intracellular Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (Baumann et al. 1996; Ghilardi et al. 1996; Bahrenberg et al. 2002). Activation of the JAK/STAT pathway results in stimulation of anorexigenic, appetite-decreasing peptides and in inhibition of orexigenic, appetite-increasing peptides. The soluble short form of LEPR presumably facilitates the transport of leptin through the blood-brain barrier (Lee et al. 1996; Tartaglia 1997; Robinson et al. 2000).

The *ob/ob* and *db/db* mice present with morbid early-onset obesity, increased adipose mass, hyperphagia, reduced energy expenditure, hyperglycemia, hyperinsulinemia, high cortisol levels, hypothyroidism, dyslipidemia, decreased body temperature and defective thermogenesis, and infertility due to hypogonadotropic hypogonadism (Friedman and Halaas 1998). In the *ob/ob* mice, these abnormalities are corrected by leptin administration (Halaas et al. 1995). In contrast, the *db/db* mice are unresponsive to endogenous and exogenous leptin (Friedman and Halaas 1998).

3.2 Mouse models involved in melanocortin signaling

The first obesity model involved in melanocortin signaling, the lethal yellow/yellow agouti (A^y) mouse strain, was described a century ago (Danforth and de Aberle 1927). Since then, several other mutations of the agouti gene: A^{vy}, A^{sy}, A^{iy}, A^{hyv} and A^{iapy}, causing yellow coat color and obesity, have been described (Yen et al. 1994). These are all promoter mutations leading to obesity, hyperphagia, hyperinsulinemia, type 2 diabetes, hypercortisolemia, hyperleptinemia, infertility and increased linear growth. The agouti locus encodes a small protein, with a signal peptide part of 22 amino acids (Bultman et al. 1992). Normally, agouti is expressed in the melanocytes of hair follicles, where it acts in hair pigmentation by blocking the action of α-melanocyte stimulating hormone (α-MSH) at the melanocortin-1 receptor (MC1R) (Lu et al. 1994). The mutations in the agouti locus result in widespread ectopic overexpression of agouti, which antagonizes MC1R in the hair follicle and MC4R in the hypothalamus, resulting in yellow coat color and in obesity, respectively (Lu et al. 1994).

Targeted deletion of the *Mc4r* gene provided evidence that the obesity syndrome in agouti mice was caused by antagonism of MC4R. *Mc4r* knockout mice present with a similar obesity syndrome as agouti mice, but have normal coat color (Huszar et al. 1997). *Mc4r* deficient mice are severly obese, hyperphagic and have accelerated longitudinal growth. Mice lacking both alleles of *Mc4r* develop a severe obesity syndrome, while heterozygous mice lacking one *Mc4r* allele have an intermediate obesity phenotype (Huszar et al. 1997). In addition to the *Mc4r* knockout mice, several other transgenic animal models elucidating the mechanisms of melanocortin signaling have been developed. For example, pro-opiomelanocortin (*Pomc*) knockout mice (Yaswen et al. 1999) and transgenic mice overexpressing a hypothalamic homolog of agouti, agouti related protein (*Agrp*) (Ollmann et al. 1997), are obese. The natural agonist of MC4R, α-MSH, is processed from the preprohormone POMC (Yaswen et al. 1999). The *Pomc* knockout mice are obese, defective in adrenal development and have yellow coat color (Yaswen et al. 1999).

The mahogany *(mg/mg)* and mahoganoid *(md/md)* mutations are natural suppressors of A^y-induced yellow pigmentation and the obesity syndrome in agouti mice (Miller et al. 1997). The *mg* and *md* genes are located on different chromosomes, but they generate the same phenotype, which suggests that the proteins are part of the same pathway. Because the mutations are not able to suppress the phenotype of *Mc1r* or *Mc4r*

knockout mice, it has been suggested that the proteins would be functional at the same level or upstream of the melanocortin receptors (Miller et al. 1997). Mahogany is a single-transmembrane protein with a large extracellular region and short cytoplasmic tail, with no signaling motifs present (Nagle et al. 1999). The exact function of mahogany is still unknown, but it has been suggested to facilitate the signaling of melanocortin receptor antagonists (Nagle et al. 1999).

The role of the melanocortin-3 receptor (MC3R) in melanocortin signaling was assessed by generating a *Mc3r* knockout mouse (Butler et al. 2000). Mice deficient for the *Mc3r* gene develop a metabolic syndrome characterized by increased fat mass, reduced lean body mass and higher feed efficiency (Butler et al. 2000; Chen et al. 2000). The mice are not significantly overweight, but they are hyperleptinemic due to increased fat mass and they have shorter bone and body length. A double knockout of the *Mc3r* and *Mc4r* genes was also created in order to understand the differences between these two mouse models (Chen et al. 2000). These double mutants were significantly heavier than the *Mc3r* or the *Mc4r* deficient mice and it was concluded that these two receptors serve non-redundant roles in energy homeostasis (Chen et al. 2000).

3.3 Carboxypeptidase E and tubby

The autosomal recessive *fat/fat* mutation in the carboxypeptidase E gene results in severe hyperproinsulinemia, i.e. elevated levels of unprocessed insulin, severe obesity, infertility and hypoadrenalism (Coleman and Eicher 1990). The carboxypeptidase E gene encodes an exopeptidase, which acts in the proteolytic maturation process of several hormones and neuropeptides (Naggert et al. 1995). The exact molecular mechanism by which the inactivation of carboxypeptidase E leads to obesity in the *fat/fat* mice is still unclear. It has been suggested that the weight gain in the *fat/fat* mice results from defects in the processing of several hypothalamic neuropeptides controlling energy intake and expenditure (Naggert et al. 1995; Leibel et al. 1997).

The autosomal recessive mutation tubby *(tub/tub)* causes maturity-onset obesity associated with hyperinsulinemia, hyperleptinemia and sensory neural defects (Coleman and Eicher 1990; Ohlemiller et al. 1995). The tubby gene encodes a protein belonging to the family of tubby-like proteins (North et al. 1997), that could represent a unique family of transcription factors (Boggon et al. 1999). The physiologic mechanism for obesity in *tubby* mice is not known. It has been suggested that the obesity in *tubby* mice might result from apoptosis of hypothalamic neural cells (Kleyn et al. 1996).

3.4 Mouse models involving transcription factors

There is growing evidence concerning the role of several transcription factors involved in the regulation of energy balance, adipogenesis, thermogenesis and consequently in the development of obesity (Nilaweera et al. 2002; Liang and Ward 2006; Stienstra et al. 2006).

The nescient helix-loop-helix 2 (NHLH2) is a transcription factor implicated in the regulation of energy balance (Nilaweera et al. 2002). Transgenic mice deficient for the *Nhlh2* gene develop severe and progressive adult-onset obesity and reduced fertility due to hypogonadism (Good et al. 1997). The obesity syndrome is similar to that seen in *Mc4r* deficient mice (Jing et al. 2004). *Nhlh2* knockout mice have a decreased number of *Pomc* expressing neurons in the arcuate nucleus, implicating a possible role for NHLH2 in the

regulation of POMC production (Nilaweera et al. 2002). Later, it was demonstrated that *Nhlh2* deficient mice have decreased levels of α-MSH due to reduced levels of prohormone convertase 1 (PC1) and PC2 enzymes, which are normally involved in posttranscriptional processing of POMC (Jing et al. 2004).

Another basic helix-loop-helix transcription factor shown to be involved in the regulation of energy balance is the homolog of the *Drosophila* single-minded 1 gene, *Sim1*. Mice homozygous for the null allele of *Sim1* gene die perinatally and lack the hypothalamic paraventricular nucleus (PVN) (Michaud et al. 1998). Heterozygous *Sim1* mice present with early-onset obesity, increased linear growth, hyperphagia, hyperinsulinemia and hyperleptinemia (Michaud et al. 2001). Heterozygous *Sim1* mice have lesions in their PVN which are suggested to cause the obesity syndrome (Michaud et al. 2001).

4 THE LEPTIN-MELANOCORTIN SIGNALING SYSTEM AND ITS DISORDERS IN HUMAN OBESITY

4.1 The leptin-melanocortin signaling pathway

Leptin is a key hormone in the physiological system regulating food intake and body weight (Friedman and Halaas 1998). Leptin is produced by adipose tissue, the circulating levels being proportional to body fat content (Considine et al. 1996). Leptin functions as a long-term peripheral satiety signal by reporting nutritional information to the brain. Within the hypothalamus of the brain, the arcuate nucleus, paraventricular nucleus, lateral hypothalamic area, ventromedial nucleus and dorsomedial nucleus are the most important areas for regulation of food intake (Hillebrand et al. 2002). In the hypothalamus leptin acts through the leptin receptor and induces the expression of the anorexic, appetite-decreasing peptides POMC and cocaine-and-amphetamine-related transcript (CART) (Spiegelman and Flier 2001). Another group of neurons expresses the orexigenic, appetite-increasing peptides neuropeptide Y (NPY) and AGRP, which are inhibited by the action of leptin (Spiegelman and Flier 2001). The leptin-melanocortin signaling pathway is depicted in Figure 1.

POMC is processed into several smaller neuropeptides by endoproteolytic cleavage (Pritchard et al. 2002). This posttranslational modification by prohormone convertases PC1 and PC2 is tissue specific, resulting in different neuropeptides in different parts of the brain. In the anterior pituitary, PC1 produces adrenocorticotrophic hormone (ACTH) and β-lipotropin, whereas in the hypothalamus the combined effect of PC1 and PC2 results in the production of α-, β- and γ-MSH and β-endorphin (Pritchard et al. 2002). Neuropeptides derived from POMC mediate the signals to a family of melanocortin receptors (MC1R-MC5R) of which MC4R and MC3R play major roles in the regulation of energy balance (Spiegelman and Flier 2001). The main agonist for MC4R and MC3R is α-MSH, although there is growing evidence that α-MSH is not the most abundant form of MSH in the brain and that other POMC derived peptides could also function as agonists (Millington et al. 2001; Harrold et al. 2003; Nickolls et al. 2003).

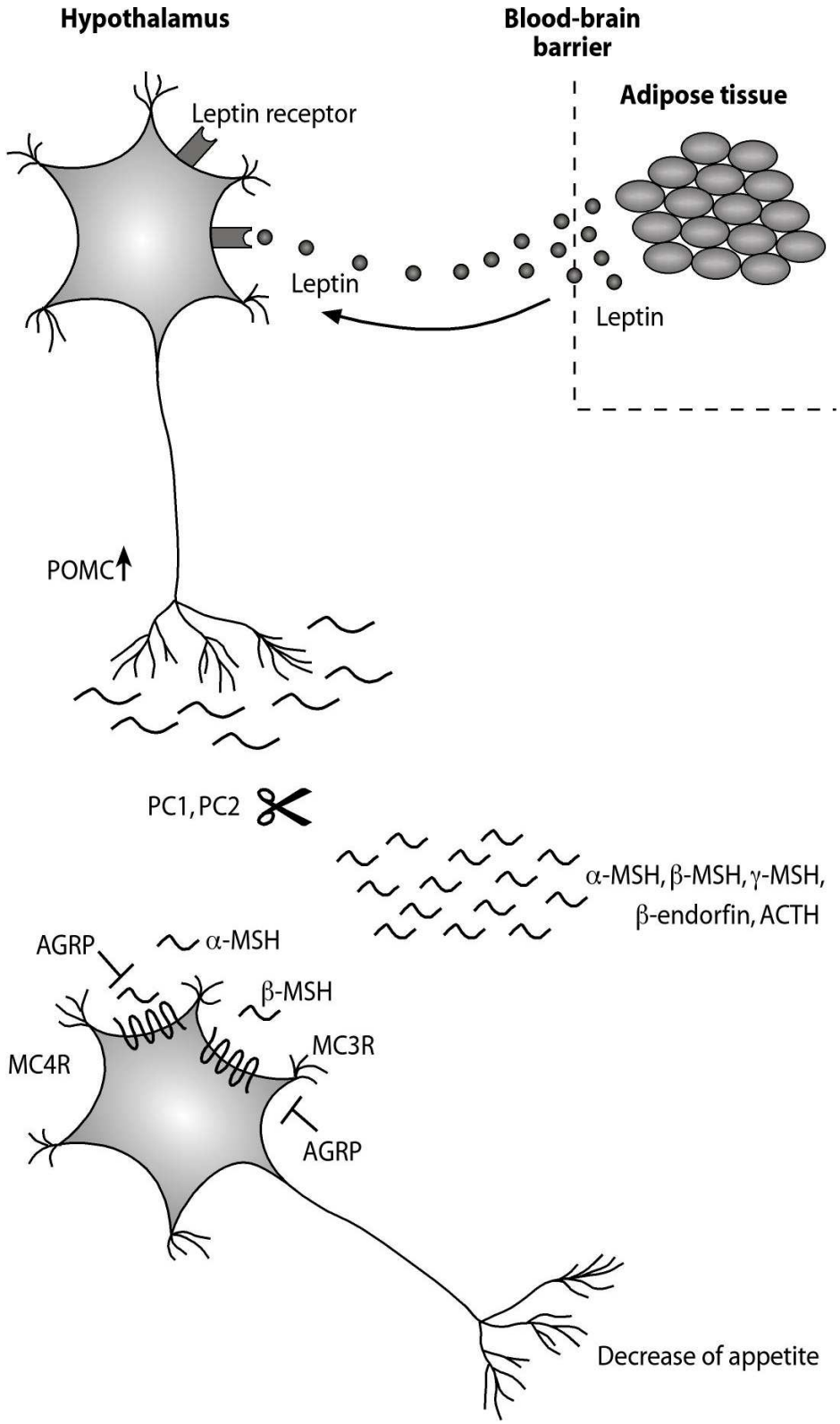

Figure 1. The leptin-melanocortin signaling pathway. Leptin is secreted from adipose tissue and is transported to the hypothalamus where it binds to the leptin receptor. Pre-prohormone POMC expression increases. The prohormone convertases PC1 and PC2 cleave POMC enzymatically into several smaller neuropeptides. Of these neuropeptides α-MSH and β-MSH play a key role in this signaling cascade. The agonists α-MSH and β-MSH bind to MC4R and MC3R and lead to the activation of the receptors. The antagonist AGRP acts by inhibiting the function of these receptors. Intracellular cAMP levels increase finally resulting in decreased appetite.

Recent data indicate that MC4R exhibits a constitutive activity upon which AGRP acts as an inverse agonist (Haskell-Luevano and Monck 2001; Nijenhuis et al. 2001; Srinivasan et al. 2004). It has been demonstrated that MC4R and MC3R are coupled to the G-protein mediated cyclic adenosine monophosphate (cAMP) pathway (Lee et al. 2001). The molecular mechanisms producing downstream effects of melanocortin signaling are not known in detail (Spiegelman and Flier 2001). However, SIM1, brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin-related kinase B (TrkB) have been indicated as potential downstream candidates of melanocortin action (Gray et al. 2006; Kublaoui et al. 2006; Gray et al. 2007).

The importance of the central melanocortin system in the regulation of energy balance and appetite has been clearly demonstrated in studies of monogenic animal models (Carroll et al. 2004). For most genes causing obesity in mouse models, human counterparts have been identified (Mutch and Clement 2006a). The disorders of the leptin-melanocortin signaling system in human obesity are presented in the following paragraphs.

4.2 Leptin

In 1997 Montague et al. reported two severely obese cousins from a consanguineous family with a homozygous frameshift mutation (delG133) in the *leptin* gene. Five further patients homozygous for the same mutation and three patients with a homozygous R105W missense mutation have been described (Farooqi and O'Rahilly 2006). All mutation carriers are characterized by severe early-onset obesity, intense hyperphagia and food-seeking behavior. The phenotype of these patients includes hyperinsulinemia, central hypothyroidism and hypogonadotropic hypogonadism. Children with leptin deficiency also have abnormalities in T-cell number and function, resulting in frequent childhood infections (Farooqi et al. 2002). Leptin deficient children and adults benefit from replacement therapy with daily subcutaneous injections of human recombinant leptin (Farooqi et al. 2002; Farooqi and O'Rahilly 2006). The major effects of leptin treatment include normalization of hyperphagia and reduction of body weight and fat mass. It has been shown that the heterozygous relatives of leptin deficient subjects have partial leptin deficiency, with significantly lower serum leptin levels than expected from their degree of obesity (Farooqi et al. 2001).

4.3 Leptin receptor

In 1998 Clement and co-workers described a homozygous mutation in the LEPR gene in a member of a consanguineous family. The mutation results in abnormal splicing of the transcript. The mutant receptor lacks transmembrane and intracellular domains but binds leptin efficiently, thus leading to high circulating levels of leptin bound to the mutant non-functional receptor. Recently, eight additional mutations of the *LEPR* gene were identified (Farooqi et al. 2007a). Six of the probands were from consanguineous families and one patient was compound heterozygous for two different mutations. Five of the mutations were nonsense and four were missense mutations. The clinical phenotype associated with *LEPR* deficiency is highly similar to the phenotype of leptin deficient patients, but somewhat less severe. In these patients, serum leptin levels were not elevated compared to equally obese subjects. Heterozygote carriers of *LEPR* mutations are characterized by increased fat mass, but their body weight is not increased (Farooqi et al. 2007a).

4.4 Pro-opiomelanocortin

Krude et al. (1998) described the first patients with congenital *POMC* deficiency. To date, six children with homozygous or compound heterozygous mutations resulting in complete loss of function of the *POMC* gene have been described (Farooqi et al. 2006). These patients present with severe early-onset obesity and ACTH deficiency. The phenotype includes pale skin and red hair color, due to the absence of α-MSH action on MC1R in pigmentation. The pigmentation phenotype may vary according to the ethnic origin of the patients, Caucasian subjects being more dependent on α-MSH in the synthesis of dark eumelanin pigment (Farooqi et al. 2006). Heterozygous mutations in *POMC* may contribute to inherited obesity (Krude et al. 2003b; Farooqi et al. 2006). A significantly higher prevalence of obesity among heterozygous carriers of *POMC* null mutations has been reported, indicating that POMC haploinsufficiency may be sufficient to predispose to obesity (Krude et al. 2003b; Farooqi et al. 2006).

Furthermore, mutations in specific parts of the pre-prohormone *POMC* gene, specifically affecting the melanocortin peptides, have been reported. The *POMC* R236G mutation, disrupting a cleavage site between β-MSH and β-endorphin, results in a fusion protein with an ability to bind to the MC4R, but with decreased ability to activate the receptor (Challis et al. 2002). This mutation is suggested to increase the risk of obesity in carriers. The *POMC* Y221C mutation affecting β-MSH was shown to be overrepresented among obese subjects and the mutant peptide had impaired ability to bind to and activate MC4R (Biebermann et al. 2006; Lee et al. 2006). In contrast to the loss of function mutations described above, the carriers of these peptide mutations have no other clinical or biochemical abnormalities besides obesity. These studies support the role of β-MSH as an important agonist of MC4R in humans. In addition, several polymorphisms of the *POMC* gene have been demonstrated to associate with common forms of obesity (Krude et al. 2003a; Rankinen et al. 2006).

4.5 Prohormone convertase 1

Three patients with congenital *PC1* deficiency have been described (Jackson et al. 1997; Jackson et al. 2003; Farooqi et al. 2007b). The first patient described presented with severe early-onset obesity, hypogonadotropic hypogonadism, postprandial hypoglycemia and hypocortisolism, due to impaired processing of POMC and proinsulin (Jackson et al. 1997). The patient was found to be a compound heterozygote for two mutations in the *PC1* gene, G593R causing a failure of autocatalytic maturation of the peptide, and a splice site mutation resulting in exon skipping and a premature stop in the catalytic domain. The second patient described was a compound heterozygote for two nonsense mutations, E250X and 213delA, resulting in loss-of-function (Jackson et al. 2003). This patient suffered from severe small intestinal absorptive dysfunction, in addition to the other phenotypic characteristics described above. The small intestinal dysfunction seen in this patient was due to abnormal processing of the prohormones progastrin and proglucagon in the enteroendocrine cells (Jackson et al. 2003). In a recent study, a third patient with congenital *PC1* deficiency was described (Farooqi et al. 2007b). The patient was homozygous for a missense mutation S307L, resulting in impaired catalytic activity of the convertase (Farooqi et al. 2007b). The phenotypic characteristics of this patient included obesity and diarrhea.

4.6 Melanocortin-4 receptor

In humans, the role of the seven transmembrane G-protein coupled MC4R in the regulation of body weight was highlighted in 1998, when two groups reported heterozygous frameshift mutations in the *MC4R* gene as a cause of dominant, severe early-onset obesity (Vaisse et al. 1998; Yeo et al. 1998). Subsequently and during the course of this study, a number of studies have reported associations between early-onset morbid obesity and *MC4R* mutations in various ethnic groups (Gu et al. 1999; Hinney et al. 1999; Sina et al. 1999; Farooqi et al. 2000; Vaisse et al. 2000; Dubern et al. 2001; Mergen et al. 2001; Hebebrand et al. 2002; Jacobson et al. 2002; Miraglia Del Giudice et al. 2002; Biebermann et al. 2003; Donohoue et al. 2003; Farooqi et al. 2003; Hinney et al. 2003; Lubrano-Berthelier et al. 2003b; Marti et al. 2003; Santini et al. 2004; Buono et al. 2005; Larsen et al. 2005; Hinney et al. 2006; Lubrano-Berthelier et al. 2006; Rong et al. 2006; Wang et al. 2006; Ochoa et al. 2007). In different studies, the prevalence of *MC4R* mutations has varied from 0.5% in obese adults (Larsen et al. 2005) to 6% in children with severe early-onset obesity (Farooqi et al. 2003). The population prevalence of *MC4R* mutations is estimated to be around 1-2.5% among people with a BMI greater than 30 kg/m^2 (Larsen et al. 2005) and thus *MC4R* deficiency represents the most common monogenic defect causing human obesity so far reported (Vaisse et al. 2000; Farooqi and O'Rahilly 2006). Theoretically, mutations in the *MC4R* promoter could also lead to obesity because of reduced transcription of the gene. The human *MC4R* gene promoter was characterized and investigated for possible abnormalities in 431 obese subjects by Lubrano-Berthelier et al. (2003a), but no promoter mutations were identified.

4.7 Melanocortin-3 receptor

MC3R is a candidate gene that has been under extensive investigation for its possible role in human obesity. The polymorphisms T6K and V81I have been identified in several studies, but in the majority of cases they were not found to be associated with obesity (Li et al. 2000; Hani et al. 2001; Wong et al. 2002; Feng et al. 2005). Three rare *MC3R* mutations have been described to be associated with obesity (Lee et al. 2002; Rached et al. 2004; Tao and Segaloff 2004; Lee et al. 2007).

4.8 Downstream targets of melanocortin signaling: SIM1, BDNF and TrkB

A few genes, including *SIM1*, *BDNF* and its receptor TrkB encoded by the gene *NTRK2*, have been implicated as potential downstream targets of melanocortin signaling (Xu et al. 2003). A patient with severe early-onset obesity was reported to have a *de novo* translocation between the chromosomes 1p22.1 and 6q16.2 disrupting the *SIM1* gene (Holder et al. 2000). In studies with mice, it was shown that both *Sim1* and *Mc4r* are expressed in the PVN and it was hypothesized that SIM1 is involved in melanocortin signaling (Michaud et al. 2001; Kublaoui et al. 2006). It was suggested that SIM1 regulates feeding rather than energy expenditure, because in contrast to *Mc4r* and *leptin* deficient mice, *Sim1* mice are not characterized by decreased energy expenditure (Michaud et al. 2001).

The neurotrophin BDNF regulates the development, survival and differentiation of neurons through its receptor TrkB (Xu et al. 2003). It has been shown that BDNF regulates eating behavior (Kernie et al. 2000) and that its expression is reduced by fasting (Xu et al.

2003). A mutation in the *NTRK2* gene was identified in an 8-year-old boy with a complex syndrome including severe obesity, impaired short term memory and developmental delay (Yeo et al. 2004). The mutation identified in the *NTRK2* gene has been suggested to impair hypothalamic signaling processes (Yeo et al. 2004; Gray et al. 2007). Recently, a patient with severe hyperphagia, morbid obesity, impaired cognitive function and memory, as well as hyperactive behavior was described (Gray et al. 2006). The patient had a *de novo* paracentric inversion in chromosome 11 disrupting the *BDNF* gene. The identification of these two patients with rare mutations in *BDNF* and *NTRK2* genes might facilitate the understanding of mechanisms regulating the hypothalamic neuronal circuits underlying regulation of energy balance (Farooqi and O'Rahilly 2006).

5 SYNDROMIC FORMS OF OBESITY

There are about 25 rare developmental syndromes that are characterized by obesity, such as Prader-Willi syndrome, Bardet-Biedl syndrome, Alström syndrome, Cohen syndrome and Borjeson-Forssman-Lehmann syndrome (for review, see Chung and Leibel 2005). These obesity syndromes are usually associated with mental retardation, dysmorphic features and organ-specific developmental abnormalities. In some cases the causative genetic defects or chromosomal abnormalities have been identified, but in most cases the molecular mechanisms underlying the syndrome are unknown. There is growing evidence of genetic heterogeneity for some of these conditions, with multiple genes producing identical phenotypes, e.g. oligogenic inheritance in Bardet-Biedl syndrome (Chung and Leibel 2005). Some of these obesity syndromes are associated with severe hyperphagia and hypothalamic dysfunction. Therefore, the elucidation of the genetic basis of these disorders could facilitate the identification of genes important for more common forms of obesity (Chung and Leibel 2005).

The most frequent of these disorders is Prader-Willi syndrome, with an incidence of one in 15,000-25,000 births (Chung and Leibel 2005). The syndrome is characterized by obesity, hyperphagia, diminished fetal activity, mental retardation and hypogonadism. The syndrome results from a loss of expression of paternal genes in the imprinted chromosomal region 15q11-13. It was suggested that in Prader-Willi syndrome, the elevated production of the gastric hormone ghrelin is responsible for increased appetite through POMC/CART and NPY/AGRP hypothalamic circuits (Cummings et al. 2002). Bardet-Biedl syndrome is characterized by early-onset obesity, rod-cone dystrophy, polydactyly, learning difficulties and renal disease (Chung and Leibel 2005). The syndrome has been associated with at least 11 chromosomal loci, with several mutations identified at each locus and evidence of complicated inheritance patterns of multiallelic transmission.

6 POLYGENIC, COMMON FORMS OF OBESITY

The present consensus maintains that common obesity is not caused by a defect in a single gene, but results from the effects of the environment and several genes together (Bell et al. 2005). In addition to the environmental contribution, the polygenic nature of obesity makes the search for obesity genes challenging. Each gene has a small effect on the phenotype and the elucidation of gene-gene and gene-environment relationships in humans is difficult (Mutch and Clement 2006a). Additionally, epigenetic mechanisms and imprinting have

been suggested to have an impact on the regulation of genes involved in growth and development (Waterland and Jirtle 2004).

The "thrifty gene" hypothesis was introduced in 1962 by James Neel. According to this theory, some genes or genetic variants would have provided selective survival advantage in times of famine and thus been maintained in the human genome during evolution. In an obesity-promoting environment these genes do not provide advantage, but instead predispose to obesity and morbidity. Most westernized societies have an environment that favors weight gain, because of the abundance of high-energy containing foods and a lack of physical activity (Mutch and Clement 2006a).

A number of studies using both genome-wide linkage and candidate gene approaches have identified a large number of genes predisposing to obesity. The latest Human Obesity Gene Map: The 2005 Update (Rankinen et al. 2006) lists 253 QTLs for obesity-related phenotypes identified in 61 genome-wide scans, 426 positive associations between obesity-related phenotypes and 127 different candidate genes. Table 3 summarizes some of the candidate genes frequently studied for association with obesity. A complete list of genes associated with obesity can be found from the Obesity Gene Map Database (http://obesitygene.pbrc.edu/).

Several of the genes of the leptin-melanocortin signaling system have been implicated as candidates for common forms of obesity. For example, *POMC* and *MC3R* have been extensively studied. Several linkage results to the genomic regions containing the *MC3R* (Borecki et al. 1994; Lembertas et al. 1997; Stone et al. 2002) and *POMC* (Hager et al. 1998; Comuzzie et al. 2001) genes have provided evidence for a role in common obesity. *POMC* has been shown to be associated with leptin levels (Hixson et al. 1999; Miraglia del Giudice et al. 2001; Suviolahti et al. 2003b) and *MC3R* with several obesity-associated traits (Boucher et al. 2002; Santoro et al. 2007).

The ectonucleotide pyrophosphatase phosphodiesterase 1 (*ENPP1*) gene is an interesting candidate gene not only for common forms of obesity, but also for insulin resistance and type 2 diabetes. *ENPP1* encodes a transmembrane glycoprotein that interacts with the insulin receptor by inhibiting its tyrosine kinase activity and subsequent signaling through the receptor (Maddux and Goldfine 2000). This in turn leads to decreased insulin sensitivity. Several studies have demonstrated linkage between the *ENPP1* locus and obesity (Arya et al. 2002; Bell et al. 2004; Fox et al. 2004; Meyre et al. 2004). Further evidence for the role of *ENPP1* is provided by studies showing association between the *ENPP1* K121Q variant and insulin resistance (Pizzuti et al. 1999; Gu et al. 2000; Frittitta et al. 2001; Abate et al. 2003), type 2 diabetes (Willer et al. 2007), as well as obesity (Barroso et al. 2003; Meyre et al. 2005a; Böttcher et al. 2006; Wan et al. 2006). The ENPP1 K121Q variant results in "gain of function" of the insulin receptor, the Q121 variant being a stronger inhibitor of the receptor (Costanzo et al. 2001). There are also studies which failed to find evidence for association between *ENPP1* and obesity, insulin resistance or type 2 diabetes (Chen et al. 2006; Gouni-Berthold et al. 2006; Lyon et al. 2006; Matsuoka et al. 2006; Weedon et al. 2006; Meyre et al. 2007). However, meta-analyses show that although the results are somewhat controversial, individuals carrying the Q121 variant have a higher risk of type 2 diabetes (Abate et al. 2005; Bacci et al. 2005; Grarup et al. 2006).

In 2006 Herbert et al. reported an association between adult and childhood obesity and a common SNP, rs7566605, located near the *INSIG2* gene (Herbert et al. 2006). The study sample consisted of 9881 adults and children, combined from case-control, family and general population cohorts from various ethnic groups. The association was found through a genome-wide association study of 86604 SNPs, under a recessive model. Other

studies investigating the role of the *INSIG2* SNP rs7566605 have been performed, but the results have not been confirmed (Dina et al. 2007; Loos et al. 2007; Rosskopf et al. 2007).

Table 3. Examples of candidate genes which have been associated with obesity-related phenotypes in multiple studies.

	Gene	Chromosome	Phenotype	References
ADIPOQ	Adiponectin	3q27	Weight, waist circumference BMI, abdominal diameter BMI, waist circumference	Menzaghi et al. 2002 Ukkola et al. 2003 Sutton et al. 2005
ADRA2A	Adrenergic receptor α-2A	10q24-q26	Skinfold thickness Skinfold thickness Abdominal total and subcutaneous fat	Oppert et al. 1995 Garenc et al. 2002 Ukkola et al. 2000
ADRB2	Adrenergic receptor β2	5q31-q32	BMI, obesity, WHR, waist and hip circumference BMI BMI, WHR	Meirhaeghe et al. 2000 Pereira et al. 2003 Lange et al. 2005
ADRB3	Adrenergic receptor β3	8p12-p11.2	BMI Onset of obesity BMI	Hao et al. 2004 Oksanen et al. 1996 Thomas et al. 2000
AGRP	Agouti related protein	16q22	BMI, body fat mass and percentage BMI, weight, body fat mass and percentage	Argyropoulos et al. 2002 Marks et al. 2004
GAD2	Glutamate decarboxylase 2	10p12	Morbid obesity, eating behavior Obesity, birth weight	Boutin et al. 2003 Meyre et al. 2005b
GHRL	Ghrelin	3p26-p25	BMI Obesity	Korbonits et al. 2002 Ukkola et al. 2001a
LEP	Leptin	7q31.3	Decrease in body weight Leptin, obesity BMI	Oksanen et al. 1997 Jiang et al. 2004 Le Stunff et al. 2000
LEPR	Leptin receptor	1p31	BMI, fat mass Fat mass, lean mass BMI	Changnon et al. 2000 Liu et al. 2004 Ross et al. 2004
NPY	Neuropeptide Y	7p15.1	BMI, WHR BMI Body weight at birth	Bray et al. 2000 Ding et al. 2005 Karvonen et al. 2000
PPARG	Peroxisome proliferative activated receptor γ	3p25	BMI, WHR BMI BMI	Kim et al. 2004 Meirhaeghe et al. 2005 Tai et al. 2004
SLC6A14	Solute carrier family 6 (neurotransmitter transporter), member 14	Xq24	Obesity, eating behavior Obesity	Durand et al. 2004 Suviolahti et al. 2003a
UCP1	Uncoupling protein 1	4q28-q31	BMI Body fat percentage Obesity	Heilbronn et al. 2000 Kim et al. 2005 Ukkola et al. 2001b
UCP2	Uncoupling protein 2	11q13	Obesity Obesity	Evans et al. 2001 Marti et al. 2004
UCP3	Uncoupling protein 3	11q13	Fat mass, lean mass, BMI, body fat percentage Body weight, resting betabolic rate	Damcott et al. 2004 Ukkola et al. 2001b

A new candidate gene for polygenic obesity, *FTO*, was identified in a recent study (Frayling et al. 2007). The *FTO* gene, having an effect on BMI, was originally identified in

a genome-wide association study searching for type 2 diabetes susceptibility genes. The association between BMI and a common gene variant (rs9939609, minor allele frequency 0.39) was replicated in several population-based cohorts. Altogether, nearly 40,000 participants were studied and the SNP rs9939609 was shown to be significantly associated with BMI in children and adults. The SNP is located within the first intron of the *FTO* gene, but the genetic and functional mechanisms underlying this association are still unclear. The *FTO* is a gene of unknown function and was cloned as a result of the identification of a mutant mouse model, fused toe (*Ft*) (Anselme et al. 2007). This gene represents the first example of a common genetic variant predisposing to obesity at the general population level (Frayling et al. 2007).

7 AN APPROACH TO A MOLECULAR GENETIC EVALUATION OF A MORBIDLY OBESE PATIENT: A CLINICAL PERSPECTIVE

The number of loci and genes associated with obesity is constantly increasing and depicts a complex view of the field of obesity genetics. Although several genes underlying monogenic and syndromic obesity have been identified, the genetic studies have mainly produced information about a large number of susceptibility genes and the findings have not always been replicated by others (Mutch and Clement 2006a). In contrast to monogenic obesity, an individual polymorphism in a single susceptibility gene may not be sufficient to cause the phenotype, but rather represents a risk factor among other genetic and environmental components predisposing to obesity (Mutch and Clement 2006a). In the case of an obese patient, combining the genetic information with information about other risk factors is a challenge for the physicians (Mutch and Clement 2006a). At the present time no systematic patient-oriented screening of gene variants predisposing for common obesity is possible.

In cases of severe early-onset obesity, in the absence of evidence for syndromic obesity, it is reasonable to investigate most of the genes encoding the leptin-melanocortin signaling system. *MC4R* is a plausible candidate worth studying, because *MC4R* deficiency represents the most common monogenic defect causing human obesity. The identification of these monogenic disorders have helped to destigmatize human obesity and allowed it to be seen as a medical condition rather than a moral failure of the patient (Farooqi and O'Rahilly 2006).

It is obvious that monogenic cases of obesity are rare. Potential therapies for affected individuals are available, as successfully demonstrated in the case of leptin deficiency (Farooqi et al. 1999). However, leptin therapy is of limited significance for public health care, as patients suffering from common obesity do not benefit from presently available agents affecting the leptin-melanocortin system. Despite intensive efforts to develop potent and selective ligands for melanocortin receptors, no specific therapies are today available for *MC4R* or *POMC* mutation carriers (Nargund et al. 2006). In *POMC* deficient children a three-month trial with an MC4R agonist was inefficient in affecting food intake or weight (Krude et al. 2003b). In contrast, these patients might benefit from the development of new small-molecule MC4R agonists. For example, analogs of β-MSH have been shown to have beneficial effects *in vitro* and *in vivo* in obese mice (Hsiung et al. 2005; Farooqi and O'Rahilly 2006). The heterozygous carriers of *MC4R* mutations may show favorable responses to pharmacotherapy with these new agents, as they have one intact, functional allele (Nargund et al. 2006).

AIMS

The aim of the present thesis study was to investigate if severe early-onset obesity in children and morbid obesity in adults can be explained by genetic variation in the melanocortin signaling pathway, with particular focus on the melanocortin receptors 3 and 4.

The specific aims were to:

1. Investigate the occurrence of monogenic forms of obesity caused by mutations in selected candidate genes in two cohorts, i.e. morbidly obese adults and children with severe early-onset obesity.

2. Identify common genetic variants, and to study previously known polymorphisms in obesity candidate genes with potential phenotypic effects leading to polygenic forms of obesity.

3. Perform *in vitro* cell studies in order to learn about structure-activity relationships of mutant MC4R.

4. Explore whether the proximal promoter regions of the *MC3R* and *MC4R* genes contain mutations or variations that are associated with obesity and whether these alterations could affect the binding of transcription factors.

5. Characterize genotype-phenotype relationships in carriers of the mutations and variants detected.

PATIENTS AND METHODS

1 STUDY SUBJECTS

1.1 Morbidly obese adults

The cohort of morbidly obese adults consisted of 252 subjects (BMI ≥ 40 kg/m^2). The morbidly obese individuals were recruited from the obesity clinic at the Department of Endocrinology, Helsinki University Central Hospital during years 1989-1995 (Oksanen et al. 1996). The cohort included 182 females and 70 males and their mean age (± SD) was 48.6 (± 9.9) years. A detailed history including data on medical history and drug treatment, as well as history of weight development, was assessed by a questionnaire. The subjects were weighed and their height and blood pressure were measured. Blood samples for DNA extraction and serum leptin, lipid, glucose and insulin were drawn after a twelve hour fast.

In study I, 48 subjects were examined for mutations in the *MC3R* gene, and the variants detected were genotyped in the entire cohort. To date, all subjects have been examined for mutations in the *MC3R* gene (partly unpublished data). In study II, all 252 subjects were examined for mutations in the *MC4R* gene. In study IV, 246 DNA samples were available for haplotype analysis of the *MC2R, MC3R, MC4R, MC5R, POMC* and *ENPP1* genes.

1.2 Children with severe early-onset obesity

Since year 2001, children with severe early-onset obesity were recruited from the Helsinki University Hospital for Children and Adolescents. The original admission criteria were a relative weight for height ratio ≥ +60% or age-specific relative BMI greater than the 98th percentile value before age of ten years. At inclusion, the patients were carefully examined, weight and height as well as WHR and blood pressure were recorded. Blood samples were drawn for DNA extraction and serum lipid, glucose and hormone measurements (thyroid-stimulating hormone, thyroxine, luteinizing hormone, follicle stimulating hormone, estradiol, testosterone etc.) after a twelve hour fast. Weight and length chart data from birth onwards were available for all children.

Fifty-six children (29 females and 27 males) were included in study II and examined for mutations in the *MC4R* gene. In study III, the number of children examined for mutations in *MC4R* was extended to 152 (66 females and 86 males). These 152 patients have also been examined for mutations in the *MC3R* and *POMC* genes (unpublished data). The entire cohort consisted of 199 children (90 females and 109 males), with a mean age (± SD) of 12.3 (± 3.9) years. All the children have been examined for mutations in the *MC4R* gene (partly unpublished data).

1.3 Background population

In studies I and II, DNA samples from 321 healthy blood donors (153 females and 159 males) served as controls for the estimation of allele frequencies in the background population. The

samples were obtained from the Finnish Red Cross Blood Transfusion Service. No data on BMI were available from these individuals.

1.4 Lean subjects

In studies III and IV, lean subjects (BMI 20-25 kg/m^2) from the national FINRISK97 cohort (Vartiainen et al. 2000) served as controls. The lean subjects were selected from the same geographical area as the adult obese subjects. In study III 447 subjects (374 females and 73 males, mean age ± SD, 52.1 ± 8.4 years), and in study IV 481 subjects (406 females and 75 males, mean age ± SD, 52.3 ± 8.3 years), served as controls.

1.5 Ethical aspects

The studies were approved by the local Ethics Review Committee of the Department of Medicine, University of Helsinki and carried out according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants or in the case of minors, from their guardians.

2 MOLECULAR GENETIC STUDIES

2.1 DNA extraction (studies I-IV)

Genomic DNA was extracted from peripheral blood lymphocytes either by a standard phenol-chloroform method (Blin and Stafford 1976), or by a salting out method with the Puregene DNA whole blood purification kit (Gentra, Minneapolis, MN, USA).

2.2 Polymerase Chain Reaction (studies I-IV)

Genomic DNA was amplified by use of Polymerase Chain Reaction (PCR) (Mullis et al. 1986). PCR was performed in varying conditions. In most cases, Amplitaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) was used. Intronic primers were designed in order to cover entire coding regions of the genes studied. If possible, promoter regions and untranslated regions (UTR) of the genes were included.

2.3 Denaturing High Performance Liquid Chromatography (studies II, III)

Mutations and polymorphisms in the *MC3R*, *MC4R* and *NHLH2* genes were searched by use of denaturing high performance liquid chromatography (dHPLC) (Donohoe 2005). DHPLC was carried out on a WAVE nucleic acid fragment analysis system HSM 3500A with a DNASep column (Transgenomic, Omaha, NE, USA). Optimal melting curves, column temperatures and eluent concentrations were calculated, separately for each PCR amplified fragment, with Wavemaker 4.1 software (Transgenomic). PCR fragments showing divergent

curve profiles in the chromatograms, indicating the presence of a heteroduplex fragment, were subsequently sequenced to identify the nucleotide substitution.

2.4 Sequencing (studies I-III)

The PCR products were purified enzymatically by use of shrimp alkaline phosphatase and exonuclease I. Sequencing reactions were performed using BigDye (Applied Biosystems) chemistry, which is based on the incorporation of di-deoxynucleotides terminating the elongation reaction (Sanger et al. 1977). The reactions were separated with an automated sequencer, ABI 377 or 3730 (Applied Biosystems), and the sequences were analyzed with Sequencher software (Gene Codes, Ann Arbor, MI, USA).

2.5 Specific detection methods for DNA alterations (studies I-III)

When mutations or polymorphisms were detected, a specific detection method was set up for each DNA alteration (Table 4). DNA samples available from relatives and control individuals were genotyped. Most of the methods set up were based on restriction enzyme digestion and electrophoretic separation of the cleavage products, either on an agarose or a polyacrylamide gel. In most cases a restriction enzyme cleavage site was naturally present at the site of the DNA alteration. In some cases artificial restriction enzyme cleavage sites were generated by primer-induced restriction analysis (Kumar and Dunn 1989). The restriction enzymes used were purchased from New England Biolabs (NEB, Beverly, MA, USA) and Fermentas (Burlington, Ontario, Canada). The *MC3R* L249V variant was detected by dHPLC and the *POMC* insertion polymorphism 73/74insSSG by heteroduplex analysis (White et al. 1992).

Table 4. Specific detection methods for DNA alterations detected.

Gene	DNA alteration	Method
MC3R (I, unpublished)	-239A>G	AlwI
	T6K	HpyCH4IV
	V81I	BseDI
	L249V	dHPLC
MC4R (II, III, unpublished)	-493delGC	Hin6I
	V103I	BpiI
	308delT	LweI
	T112M	HpyCH4IV
	S127L	MlyI
	M200V	NcoI
	I226T	BoxI
	I251L	Kpn2I
	P299H	AlwI
	1059C>T	BstNI
NHLH2 (III)	L32L (96C>T)	Alw21I
POMC (unpublished)	73/74insSSG	Heteroduplex analysis
	E188G	MspI
	8469G>C	NmuCI

2.6 SNP genotyping by SEQUENOM massARRAY (study IV)

To confirm that the SNPs with no prior frequency information were polymorphic (had a frequency over 10%), 15 control individuals and a DNA-pool of 130 control samples were genotyped. In order to confirm Mendelian inheritance, 57 nuclear families including a father, mother and child were genotyped. Reproducibility of genotype data was guaranteed by genotyping 2% of all samples in duplicates. SNPs were excluded from further genotyping if they were in complete LD with an adjacent SNP. Assay validity criteria for acceptance of SNPs for genotyping were: 1) no discordant results in duplicates, 2) allele distributions in Hardy-Weinberg equilibrium, 3) no Mendelian errors in the nuclear families and 4) genotyping success rate > 90%.

SNPs were genotyped using the homogenous MassEXTEND® assay on the MassARRAY® system (SEQUENOM Inc., San Diego, CA, USA) according to the manufacturer's instructions (Jurinke et al. 2002). The method is based on allele-specific primer extension, resulting in extension products each having a unique molar mass. The extension products having distinct masses can be detected by use of a matrix assisted laser desorption/ionization time-of-flight mass spectrometry.

2.7 Bioinformatics and computational biology (studies I-IV)

Several internet search tools, databases and free software, as well as commercial programs were utilized during this study. Particularly valuable were the Obesity Gene Map Database (http://obesitygene.pbrc.edu/), National Center for Biotechnology Information NCBI (http://www.ncbi.nlm.nih.gov/) and UCSC Genome Bioinformatics (http://genome.ucsc.edu/) sites. In all studies, PCR primers were designed with the Primer3 program (Rozen and Skaletsky 2000). Webcutter 2.0 (http://www.firstmarket.com/cutter/cut2.html) and RestrictionMapper (http://www.restrictionmapper.org/index.htm) programs were used when setting up the specific detection methods for DNA alterations. DNA alterations located in non-coding regions were analyzed by MatInspector (Quandt et al. 1995), TFSearch (Heinemeyer et al. 1998), TESS (Schug and Overton 1997), ConSite (Sandelin et al. 2004) and Con Real (Berezikov et al. 2004) programs. In study IV, human and mouse sequences were compared using Pipmaker (Schwartz et al. 2000) and Vista (Frazer et al. 2004) software.

3 STATISTICAL METHODS (studies I-IV)

Statistical analyses were mainly performed with NCSS 2000 software (NCSS, Kaysville, Utah, USA). Differences in allele and genotype frequencies between groups were tested by chi-square or Fisher's exact tests, where appropriate. In study I, Mann-Whitney rank-sum test was used to analyze differences between group means of metabolic parameters. In study IV, Genepop v3.4 Option 2 software (Raymond and Rousset 1995) was used to test the LD between adjacent SNPs. Both D' and r^2 values were calculated in order to test pairwise LD between the SNPs. Haplotype structure and frequencies were estimated using Haploview software (Barrett et al. 2005). The association of each SNP genotype with obesity and diabetes was assessed using logistic regression analysis, by adjusting for age and sex. A type 1 error rate of 0.1 for multiple testing was controlled for by using the false discovery rate method (Benjamini and Hochberg 1995).

4 FUNCTIONAL ANALYSES

4.1 Preparation of plasmid constructs (studies II, III)

Site-directed mutagenesis and cloning techniques were utilized in creating constructs for the functional analyses. In study II, *MC4R* mutations T112M, S127L, M200V (unpublished) and I226T were introduced into two constructs; a human *MC4R* cloned in pcDNA3 (Invitrogen, Carlsbad, CA, USA) and a HA-tagged MC4R green fluorescent protein fusion protein (HA-MC4R-GFP) encoded in pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA, USA). The constructs were kindly provided by Dr. J.S. Flier (Shinyama et al. 2003). The mutations were incorporated with a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA).

For study III, human *NHLH2* cloned in pBluescript II (Stratagene) was kindly provided by Dr. I.R. Kirsch (Lipkowitz et al. 1992). For gel shift assays, an EcoRI (NEB) and XbaI (NEB) restricted complementary DNA fragment of *NHLH2* was sub-cloned by ligation in-frame to pFLAG-CMV2 (Sigma-Aldrich, Inc., St. Louis, MO, USA). For reporter gene experiments the Mc4rwt and Mc4rdelGC constructs were created by amplifying the *MC4R* minimal promoter region (Mc4r-130/+10, according to (Lubrano-Berthelier et al. 2003a) by PCR. By amplifying heterozygous patient DNA, both mutant and wild-type (wt) promoter constructs were produced simultaneously. The PCR primers contained NheI (NEB) and XhoI (NEB) restriction sites enabling the ligation of the PCR fragments to pGL3-basic (Promega, Madison, WI, USA). The integrity of all constructs was verified by sequencing.

4.2 Cell culture and transfection: *in vitro* expression studies (studies II, III)

The functional properties of mutant MC4Rs were studied in 293T cells (American Type Culture Collection, ATCC, Manassas, VA, USA). Nuclear cell extracts containing NHLH2 protein was prepared in COS-1 cells (ATCC) and the *MC4R* promoter was studied in HeLa (ATCC), COS-1 and LβT2 cells (provided by Dr. P.L. Mellon) (Thomas et al. 1996). The 293T cells were maintained in Dulbecco's Modified Eagle Medium, high glucose, supplemented with 10% (vol/vol) fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, at 37ºC in a humidified atmosphere containing 5% CO_2. The cell culture conditions were similar for all cells, but media concentrations were adjusted according to the requirements of each cell type.

MC4R signaling was studied by transiently transfecting 293T cells with wt or mutant MC4R and a cAMP responsive thyrotropin releasing hormone (TRH) luciferase reporter (TRH-Luc) (Harris et al. 2001) (Figure 2A). Transfections were performed with Lipofectamine Reagent (Invitrogen) in OPTI-MEM I Reduced Serum Medium (Invitrogen). After transfection and overnight incubation cells were stimulated with increasing amounts of the MC4R agonists α-MSH (Sigma-Aldrich), β-MSH (Phoenix Pharmaceuticals, Belmont, CA, USA) or γ_1-MSH (Phoenix Pharmaceuticals) and incubated for 5 h at 37°C, 5% CO_2. To evaluate the effect of the MC4R antagonist AGRP (83-132) (Phoenix Pharmaceuticals) on the mutant receptors, cells were pretreated with increasing concentrations of AGRP for 30 min and incubated for 5 h in a constant 100 nM α-MSH concentration. The luciferase activity was measured with an EG&G Berthold LB 9501 Luminometer (Berthold Technologies, Bad Wilbad, Germany) (Bjørbæk et al. 1998). A β-galactosidase expression vector under control of a CMV promoter was included in all transfections as an internal control. All values were

normalized for transfection efficiency by determination of ß-galactosidase activity with the Galacto-Light kit (Applied Biosystems).

The functional properties of variant receptor M200V was studied in 293H cells (ATCC) in a similar way as described above. FuGENE-6 (Roche Molecular Biochemicals, Mannheim, Germany) was used as transfection reagent and luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega). The system allows the determination of firefly and Renilla luciferase activities from the same cell lysates. Normalization for transfection efficiency was performed by determination of Renilla luciferase activity of the internal pRL- control vector (Promega).

In study III, nuclear extracts of NHLH2 were prepared by transfecting COS-1 cells with the NHLH2 construct by FuGENE-6 Transfection Reagent (Roche Molecular Biochemicals). Cells were collected 48 h after transfection, washed once with phosphate buffered saline (PBS) and lysed. In the *MC4R* promoter assays (Figure 2B) HeLa, COS-1 or LβT2 cells (Windle et al. 1990) were transfected with luciferase reporter Mc4rwt or Mc4rdelGC. The cells were lysed 48 h after transfection with reporter lysis buffer (Promega) and the luciferase activity was determined with reagents from Promega and a Luminoscan Ascent reader (Thermo LabSystems, Beverly, MA, USA). An internal control vector pCMVβ (Clontech, Palo Alto, CA, USA) was included in all transfections and the values were normalized for transfection efficiency by enzymatic determination of β-galactosidase activity (Kotaja et al. 2002).

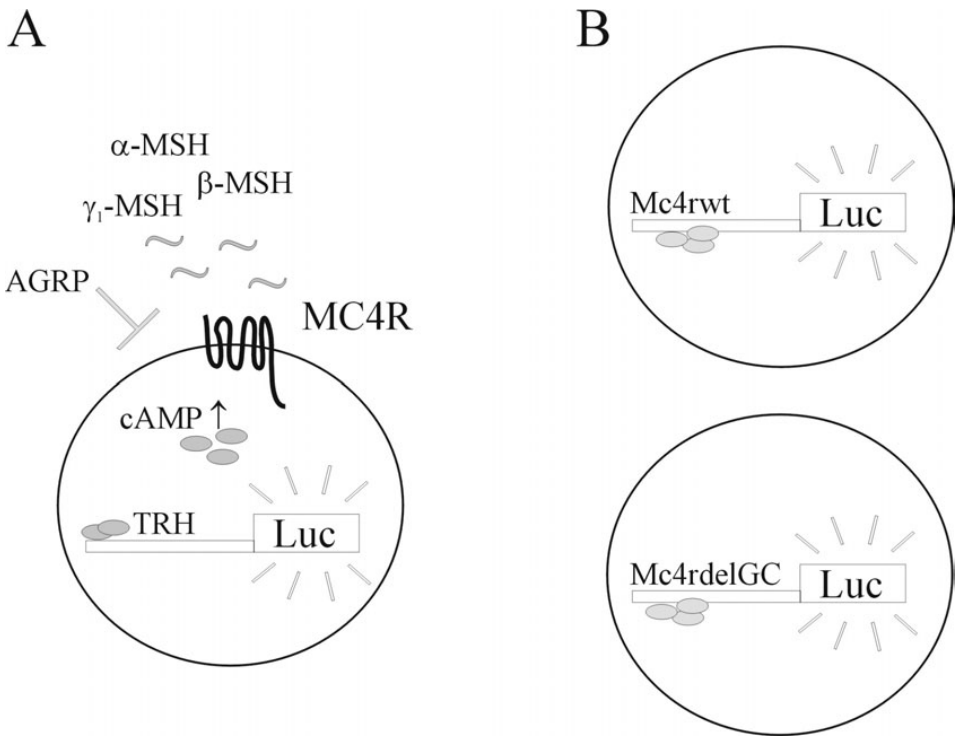

Figure 2. Principles of the *in vitro* expression methods used. A) 293T or 293H cells were transiently transfected with wt or mutant MC4R and a cAMP responsive TRH-Luc reporter construct. The cells were stimulated with MC4R agonists α-MSH, β-MSH or γ_1-MSH. The stimulation of MC4R leads to the increase of intracellular cAMP levels. Cyclic-AMP binds to the TRH promoter and turns on the luciferase activity of the reporter construct, which can be determined luminometrically. Binding of the MC4R agonist AGRP was studied by measuring luciferase activity of α-MSH stimulated cells that were pretreated with AGRP. B) The -493delGC deletion in the *MC4R* promoter was studied by transiently transfecting HeLa, COS-1 or LβT2 cells with luciferase reporter constructs containing wt (Mc4rwt) or mutant (Mc4rdelGC) promoters. The luciferase activity produced by the Mc4rwt or Mc4rdelGC promoter was determined.

4.3 Western blotting (studies II, III)

To ensure that the transfection efficiency was adequate for the following experiments, the wt and mutant MC4R proteins were detected by standard Western blotting procedures. In study II, a monoclonal anti-HA antibody (Roche Molecular Biochemicals) recognizing the HA-tag included in the MC4R constructs was used. In study III, the expression efficiency of transfected NHLH2 was detected using a monoclonal anti-FLAG antibody (Roche Molecular Biochemicals).

4.4 Immunofluorescence and Enzyme Linked Immunosorbent Assay (study II)

To evaluate whether the MC4R mutant receptors were functionally impaired due to intracellular retention, immunofluorescence and enzyme linked immunosorbent assay (ELISA) experiments were performed in 293T or 293H cells to detect HA-MC4R-GFP at the cell surface. Cells were plated on Poly-D-lysine (Sigma-Aldrich) coated Lab-Tek Chamber Slides (Nalge Nunc International, Naperville, IL, USA) or on 24-well plates and transfected with mutant and wt vectors. For immunofluorescence cells were washed once with cold PBS, fixed with 3% paraformaldehyde in PBS and mounted with VECTASHIELD Mounting Medium with DAPI (4',6-diamidino-2-phenylindole) (Vector Laboratories, Burlingame, CA, USA) or Calbiochem MOWIOL 4-88 Reagent (Merck KgaA, Darmstadt, Germany). Cells were visualized on a fluorescence microscope and images were taken with a digital camera. ELISA was performed as described by Shinyama et al. (2003). Briefly, monoclonal high affinity anti-HA-peroxidase antibody (Roche Molecular Biochemicals) and POD substrate (Roche Molecular Biochemicals) were used in the peroxidase-mediated ELISA and the absorbance was measured at 450 nm.

4.5 Electrophoretic Mobility Shift Assay (studies I, III)

The binding of proteins to specific regions in *MC3R* and *MC4R* promoters were studied by electrophoretic mobility shift assays (EMSA). Nuclear proteins for gel shifts with the *MC3R* promoter were prepared from cell lines expressing GATA-4 and GATA-6 (MSC-1, mouse Sertoli), GATA-3 (Jurkat, T-cell lymphoma) and no known GATA proteins (NIH 3T3, fibroblast). Extracts of cells trasfected with NHLH2 were used for experiments with the *MC4R* promoter. All cell extracts were prepared as described by Andrews and Faller (1991). Probes were annealed and labeled with [γ-^{32}P]ATP as described in detail in studies I and III. In study I, binding reactions were performed as described by Martelin et al. (2000). GATA antibodies for supershift experiments were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). In study III, binding reactions were performed in binding buffer (10 mM Tris-HCl pH 8.0, 40 mM KCl, 0.1% Nonidet P-40, 6% glycerol, 1 mM dithiothreitol, 1:100 protease inhibitor cocktail). The labeled probe was added to the reaction and incubated at 4°C for 30 minutes. In both studies the samples were separated on 4% polyacrylamide gels and visualized by autoradiography.

RESULTS

1 GENETIC VARIATIONS IDENTIFIED IN FINNISH OBESE PATIENTS

1.1 The melanocortin-3 receptor gene

In study I, 48 morbidly obese adults were screened for mutations in the *MC3R* gene by sequencing. The previously described common missense mutations in the coding region, T6L and V81I, and a 5'UTR variant -239A>G were detected in the *MC3R* gene (Li et al. 2000; Hani et al. 2001). Additionally, four previously unknown variants -939G>C, -911G>A, -803T>C and -373G>T were detected in the 5'UTR.

Allele frequencies of genetic variants -239A>G, T6L and V81I were determined in the entire cohort of morbidly obese subjects and in a background population sample of healthy blood donors (Table 5). Allele frequencies did not differ significantly between the groups studied. The T6L and V81I variants were co-inherited in all but 3 cases, indicating a high level of LD. Individuals carrying the wt alleles (T6/V81) had lower insulin-glucose ratio (3.1 ± 0.2 vs. 4.1 ± 0.4, $p<0.05$) and lower leptin (ng/ml) levels (38.5 ± 1.5 vs. 46.4 ± 3.6, $p<0.05$) compared to those carrying the heterozygous and homozygous variant alleles.

To date, the entire cohort of morbidly obese adults (n=252) and 152 children with severe early-onset obesity have been investigated for mutations in the *MC3R* gene. In addition to the variants described above, a silent nucleotide substitution 354G>C (A118A), a missense mutation L249V and a nucleotide substitution 1091G>A in the 3'UTR were detected among morbidly obese adults. The missense mutation L249V was not detected in the background population (n=312). Allele frequencies of variants -239A>G, T6L and V81I were also determined in the cohort of children with severe early-onset obesity (Table 5). Furthermore, previously unknown variants -335G>A, -135insCT and 327G>A (P109P) were detected among patients with severe early-onset obesity.

Table 5. Minor allele frequencies of *MC3R* variants in morbidly obese adults, children with severe early-onset obesity and a background population sample. There were no significant differences between the three groups.

MC3R variant	Morbidly obese adults (n=252)	Children with severe early-onset obesity (n=152)	Background population (n=312)
-239A>G	0.11	0.10	0.13
T6L	0.09	0.14	0.11
V81I	0.08	0.12	0.10

1.2 The melanocortin-4 receptor gene

A total of 252 morbidly obese adults and 199 children with early-onset obesity were investigated for mutations in the *MC4R* gene by dHPLC and direct sequencing (studies II, III and unpublished data). Several heterozygous mutations and polymorphisms were detected in the *MC4R* gene (Figure 3 and Table 6). In studies II and III, the missense mutations V103I and S127L, a deletion -439delGC in the putative promoter region and a nucleotide substitution 1059C>T in the 3'UTR of *MC4R* were identified among severely obese children. Among adult morbidly obese patients, the variants V103I, T112M, I226T, and I251L were detected. In addition, four common polymorphisms (-1042C>T, -1005C>T, -896C>T and

-719G>A) were found in the 5' flanking region of *MC4R* in all cohorts studied. At a later time point, the number of patients in the cohort of children with severe early-onset obesity was extended to 199 (unpublished data). The following genetic variants were detected among the newly recruited patients: nucleotide substitutions -483C>T and -178A>C in the 5'UTR, a deletion 308delT and missense variants V103I, T112M, S127L, M200V and P229H in the coding region of the *MC4R* gene. All mutations and polymorphisms in the *MC4R* gene were detected in heterozygous form.

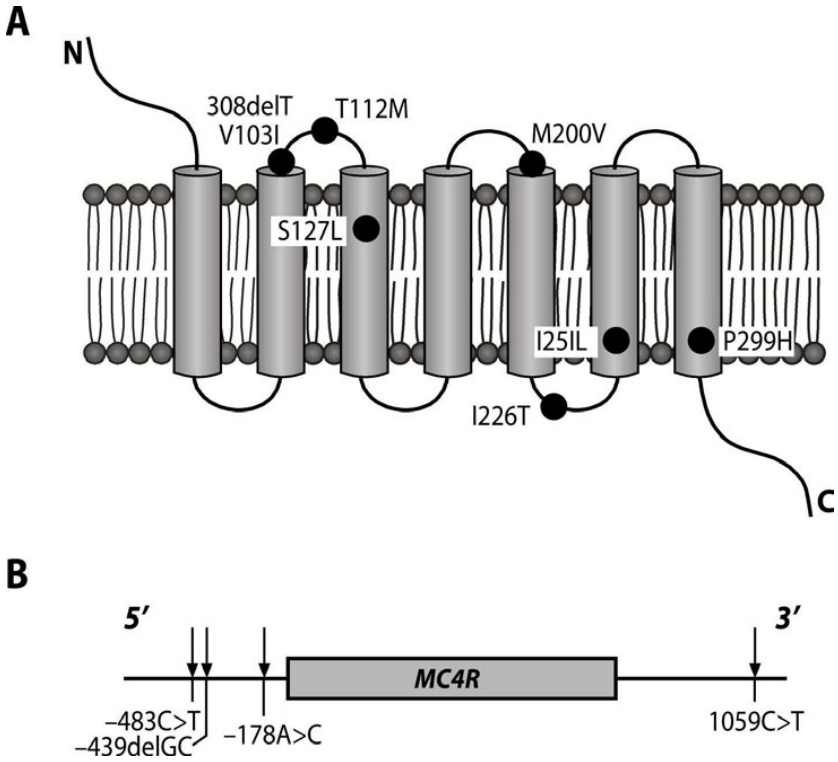

Figure 3. Schematic drawing presenting the location of mutations and polymorphisms detected in the *MC4R* gene. A) Genetic variants in the coding region of the MC4R. B) Variants located in the promoter region, 5'UTR and 3'UTR of the *MC4R* gene.

Table 6. *MC4R* mutations and polymorphisms identified in heterozygous form in children with early-onset obesity and morbidly obese adults.

MC4R variant	Children with severe early-onset obesity (n = 199)	Morbidly obese adults (n = 252)	Background population (n = 321)
-483C>T	1	0	ND
-439delGC	2	0	1[a]
-178A>C	1	0	ND
308delT	1	0	0
V103I	3	1	9
T112M	2	3	0
S127L	2	0	0
M200V	1	0	0
I226T	0	1	3
I251L	0	1	8
P299H	1	0	0
1059C>T	1	0	ND

ND, not determined. [a]The deletion was not present in 447 lean controls.

Three of the genetic variants identified can be regarded as mutations that affect the function of the receptor (308delT, S127L, P299H). The deletion -439delGC in the *MC4R* promoter affects a basic helix-loop-helix (bHLH)-binding sequence and might have an impact on the transcriptional activity of the gene. The other genetic variants identified are proposed to be polymorphisms with no detectable effect on the activity of the gene or on the function of the receptor.

1.2.1 Mutations in the coding region of *MC4R*

A novel deletion, 308delT, was identified in a 13.3-year-old girl (unpublished data). The 1-basepair deletion at nucleotide position 308 causes a frameshift at codon 103, and introduces a stretch of four new amino acids (Ala-Ser-Pro-Tyr) and a premature stop at codon 107. The 308delT index patient had early-onset obesity and early-onset type 2 diabetes. Her abnormal weight-gain began before the age of one year (Figure 5A) and type 2 diabetes was diagnosed at the age of 13 years. The patient's glycosylated hemoglobin was 7.3% (normal range 4-6%) and she was negative for autoantibodies associated with type 1 diabetes. The index patient's mother was shown to be a carrier of the 308delT mutation (Figure 4). The mother's BMI was > 25 kg/m^2 and she was severely obese in childhood. The index patient had familial predisposition for type 2 diabetes, as her father, paternal grandfather and maternal grandmother, were affected by the disease.

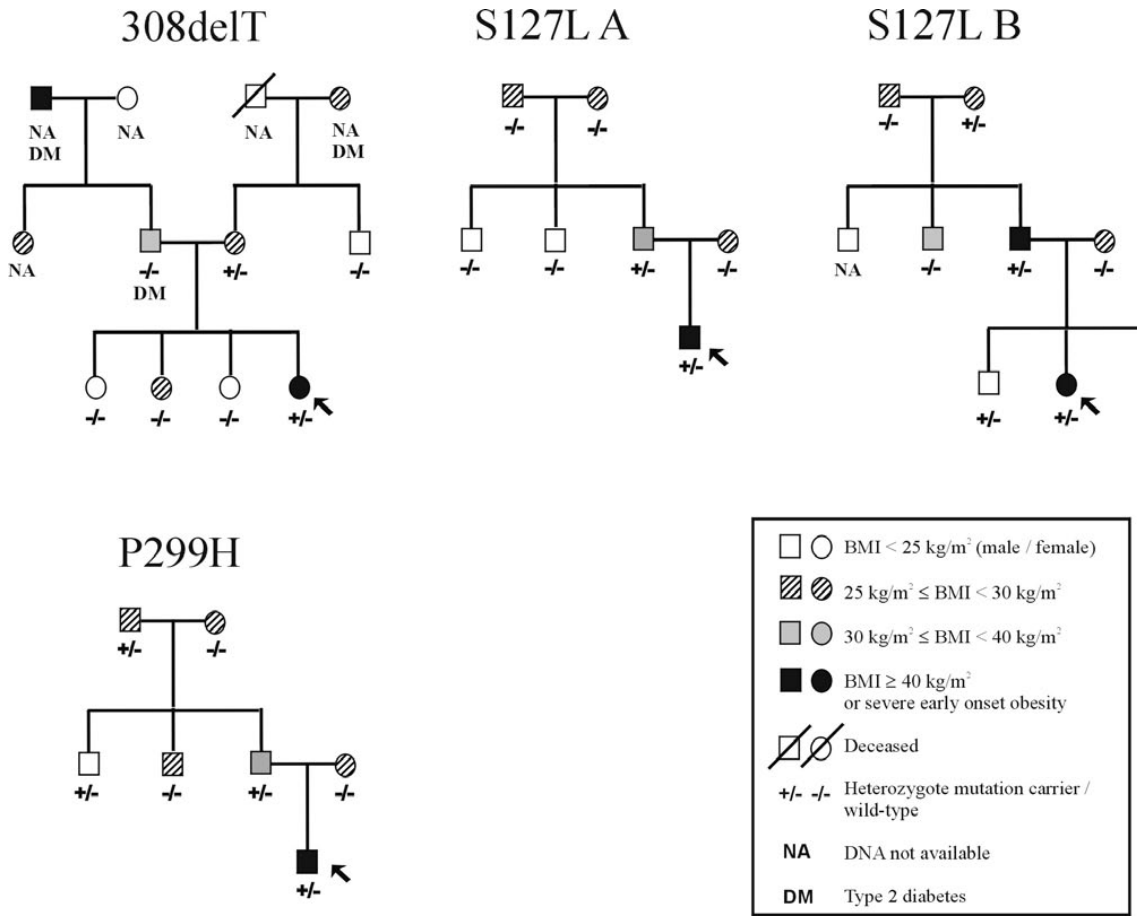

Figure 4. Pedigrees of families with *MC4R* mutations. The index patients are indicated by arrows. *Modified from figure 1A, study II.*

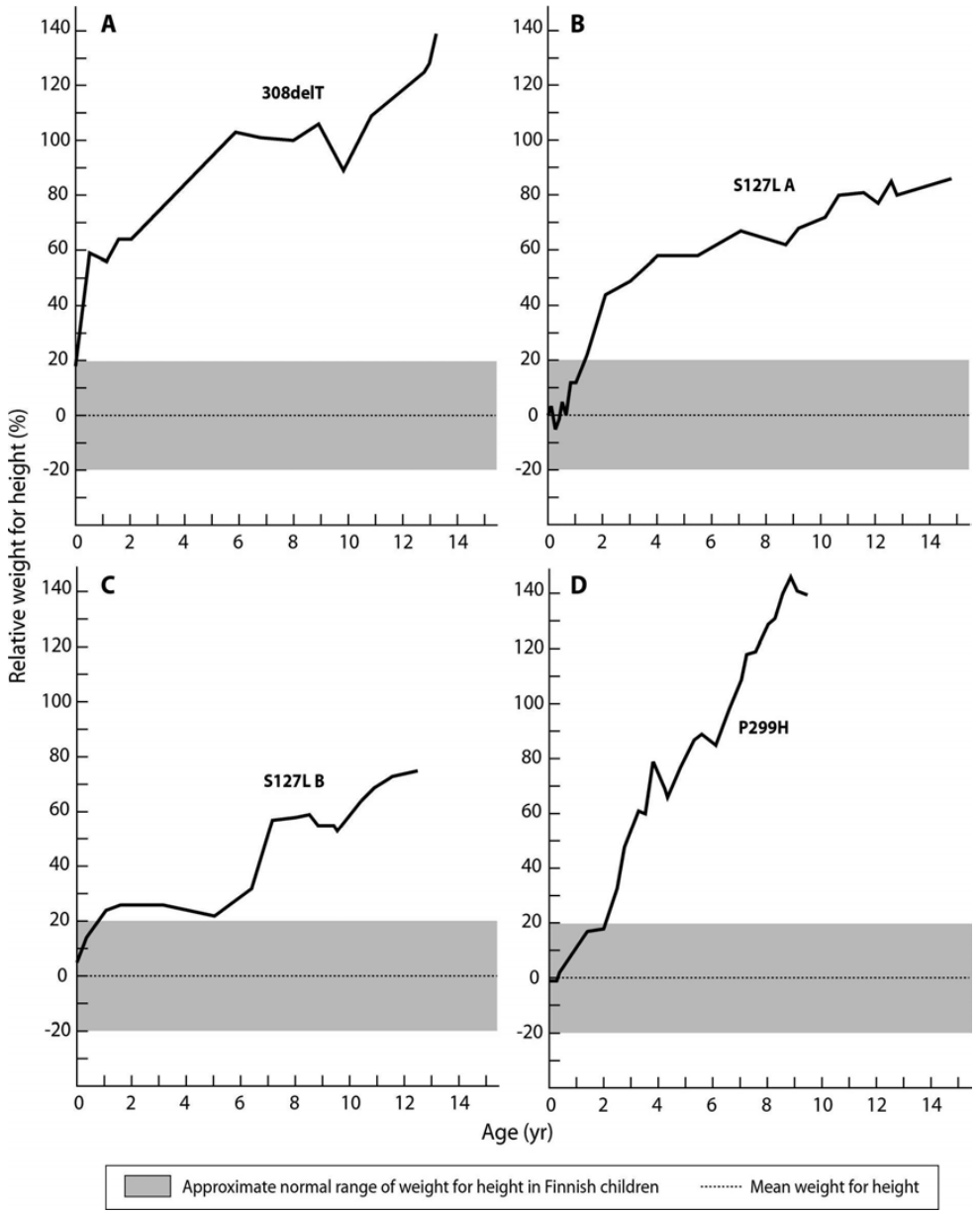

Figure 5. Charts presenting the weight development of index patients with *MC4R* mutations. A) 308delT, B) S127L index patient A, C) S127L index patient B, D) P299H. *Modified from figure 1B, study II.*

A missense mutation P299H was identified in a 9.3-year-old boy (unpublished data). The mutation has been described and the functional properties investigated previously (Lubrano-Berthelier et al. 2003b). Lubrano-Berthelier and co-workers demonstrated that the P299H mutation leads to intracellular retention of the receptor. The index patient's abnormal weight gain began in the second year of life (Figure 5D). The P299H mutation was also detected in the index patient's obese father, the father's lean brother and the overweight paternal grandfather (Figure 4).

The S127L mutation was first detected in a 14.8-year-old boy (study II) and later the same mutation was identified in a 12.5-year old girl (unpublished data). Both carriers of the S127L mutations were also carriers of the *MC4R* polymorphism V103I. The receptor is transported normally to the cell membrane, but signaling through the mutated receptor is impaired (study II and Lubrano-Berthelier et al. 2003b). Index patient A's abnormal weight gain began in the second year of life (Figure 5B). He had normal glucose tolerance, but an

oral glucose tolerance test (OGTT) revealed marked hyperinsulinemia (detailed information in study II) and he presented with acanthosis nigricans in the neck and axillae indicating insulin resistance. In family A, only the proband's moderately obese father proved to be positive for the S127L mutation, which could not be found in the grandparents (Figure 4). Index patient B started abnormally gaining weight at the age of five years (Figure 5C). In family B, the index patient's lean brother, morbidly obese father and overweight paternal grandmother were shown to be carriers of the S127L mutation (Figure 4).

Table 7 presents the clinical characteristics of *MC4R* mutation carriers.

Table 7. Clinical features and hormone levels in the six index patients carrying *MC4R* mutations.

Variable	308delT	P299H	S127L Index patient A	S127L Index patient B	-439delGC Index patient A	-439delGC Index patient B
Sex	Female	Male	Male	Female	Female	Male
Age, years	13.3	9.3	14.8	12.5	13.8	10.1
Weight, kg	127.6	73.0	111.2	94.5	103.5	74.2
Height, cm (+SDS)	166.7 (+1.1)	136.5 (+0.2)	174.0 (+0.8)	167.5 (+1.9)	169.5 (+1.5)	154.2 (+2.4)
BMI, kg/m^2	45.9	39.2	36.7	33.6	33.5	31.2
fP-Glucose, mmol/l [a]	7.2	5.0	4.7	5.8	5.6	4.9
fS-Insulin, mU/l [b]	39.0	35.6	33.0	22.8	14.0	38.0
S-Leptin, µg/l	38.4	62.8	21.0	29.1	32.6	51.4
Blood pressure (systolic/diastolic), mm Hg	144/88	134/70	123/71	140/70	136/79	112/65
fP-Cholesterol, mmol/l [c]	5.3	3.8	4.2	4.2	5.0	2.7
fP-Chol-HDL, mmol/l [d]	1.0	1.03	1.31	1.05	1.11	0.75
fP-Chol-LDL, mmol/l [e]	2.95	2.38	2.31	2.52	3.26	1.59
f-Triglycerides, mmol/l [f]	2.95	0.86	1.13	1.39	1.36	1.8
P-ALAT, U/l [g]	163	15	64	19	18	227
P-Urate, µmol/l [h]	523	195	505	310	292	396
S-TSH, mU/l [i]	2.20	1.99	1.28	3.93	3.3	1.7
S-T4-free, pmol/l [j]	14.0	15.0	14.0	15.0	11.0	14.0
S-Estradiol, nmol/l	0.09	-	-	0.06	0.06	-
S-LH, IU/l	5.4	<0.1	0.8	2.2	1.5	<0.1
S-FSH, IU/l	5.8	0.1	1.2	6.2	3.6	0.3
Testosterone, nmol/l	-	0.3	7.0	-	-	0.1

Abbreviations used in the table: SDS, standard deviation score; fP, fasting plasma; fS, fasting serum; S, serum; HDL, high density lipoprotein; LDL, low density lipoprotein; f, fasting; ALAT, alanine aminotransferase; TSH, thyroid-stimulating hormone; T4, thyroxine; LH, luteinizing hormone, FSH, follicle stimulating hormone.
Normal range: [a] 3.8-7 nmol/l, [b] 2.3-26 mU/l, [c] <5mol/l, [d] 0.93-1.94 mol/l, [e] 1.6-3.6 mol/l, [f] children 13-months to 9-years 0.31-1.46 mol/l, children 10-13-years 0.27-1.64 mol/l, boys 14-15-years 0.38-1.86 mol/l, [g] <40 U/l, [h] 120-330 µmol/l, [i] 0.4-5 mU/l and [j] girls 11-15-years 10-19 pmol/l, boys 6-10-years 10-22 pmol/l, boys 11-15-years 12-20 pmol/l.

1.2.2 A deletion in the *MC4R* promoter, -439delGC

In studies II and III, the deletion -439delGC was identified in two unrelated children with severe early-onset obesity. The presence of the -439delGC deletion was investigated in 454 normal weight (BMI 20-25 kg/m^2) subjects. No carriers of this mutation were found among the lean controls.

The mutation was first found in a 13.8-year-old girl with a BMI of 33.5 kg/m^2 and later the same mutation was identified in a 10.1-year-old boy with a BMI of 31.2 kg/m^2. Both mutation carriers had abnormal weight gain from the age of four years onwards. Detailed information about the weight development of these children can be found in study III. In family A, the index patient's 35-year-old half-sister was a carrier of the -439delGC mutation. She had a history of childhood obesity and is currently obese (BMI 31.9 kg/m^2). In family B, the index patient's 11.8-year-old obese brother (BMI 27.6 kg/m^2), lean father (current BMI 23.1 kg/m^2, overweight in infancy) and the father's lean sister (current BMI 23.7 kg/m^2, overweight as a child until reaching puberty) were carriers of the mutation. (Pedigrees of the families are shown in study III).

1.3 The nescient helix loop helix 2 gene

In study III, the possible role of transcription factor NHLH2 in obesity was investigated by screening the gene for mutations in 152 subjects with severe early-onset obesity. No mutations were detected in the *NHLH2* gene. However, one silent nucleotide substitution 96C>T (L32L) was found in two children with severe early-onset obesity.

1.4 The pro-opiomelanocortin gene

The possible impact of the *POMC* gene on obesity was studied by sequencing the gene in 91 children with severe early-onset obesity (unpublished data). The variants detected are presented in Figure 6 and Table 8.

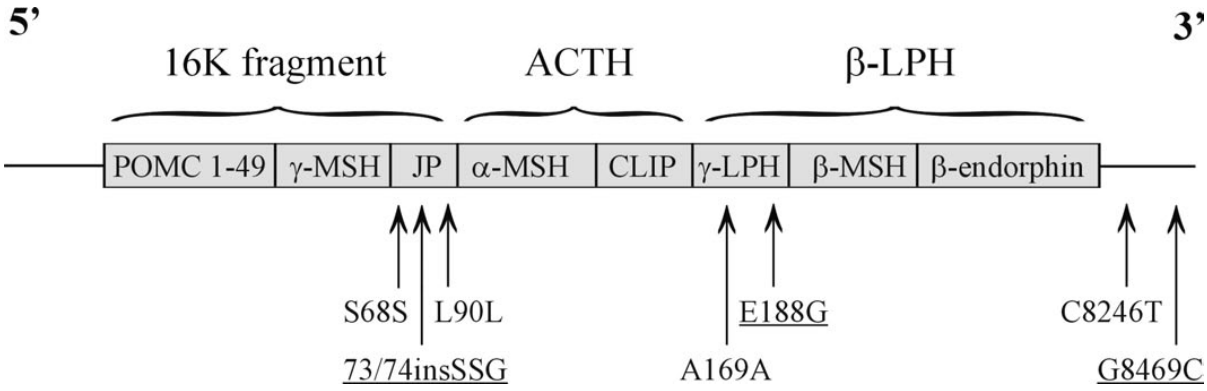

Figure 6. The structure of the *POMC* gene and the locations of the variants detected. The variants chosen for further determination of allele frequencies are underlined.

Table 8. Variants detected in the *POMC* gene in children with severe early-onset obesity (n=91).

Nucleotide	Amino acid / location	Heterozygous	Homozygous
C7662T	S68S	11	-
7676/7677ins9bp	73/74insSSG	13	1
C7726T	L90L	1	-
C7965T	A169A	11	-
A8021G	E188G	2	-
C8246T	3'UTR	19	-
G8469C	3'UTR	1	-

Three variants were chosen for determination of allele frequencies in 248 adult morbidly obese subjects and 191 subjects from the background population (Table 9). No significant differences in allele frequencies were detected between the groups studied.

Table 9. Minor allele frequencies of *POMC* variants in morbidly obese adults, children with severe early-onset obesity and a background population sample.

POMC variant	Morbidly obese adults (n=252)	Children with severe early-onset obesity (n=91)	Background population (n=191)
73/74insSSG	0.042	0.082	0.068
E188G	0.014	0.011	0.003
G8469C	0.016	0.05	0.018

2 FUNCTIONAL STUDIES OF *MC4R* AND *MC3R* GENES

2.1 Functional properties of mutant melanocortin-4 receptors *in vitro*

The ability of MC4R to generate cAMP in response to increasing concentrations of α-MSH was studied by co-transfecting 293T or 293H cells with wt or mutant MC4R and a cAMP responsive TRH-luciferase promoter construct. Receptor mutations T112M, S127L, I226T (study II) and M200V (unpublished data) were studied. The luciferase assay revealed impaired response of the S127L mutant receptor to α-MSH (study II, Figure 2A). In study II, the mutant receptors T112M, S127L and I226T were also tested for β-MSH and γ_1-MSH. The activity of the S127L mutant receptor was impaired for all peptides tested, while the other mutants gave responses similar to the wt receptor (study II, Figure 2B-C). No difference was detected between the different mutants and the wt receptor when tested for inhibition by AGRP (study II, Figure 2D-E). The activity of the M200V receptor in response to α-MSH (Figure 7A) and AGRP (Figure 7B) was similar compared to that of the wt receptor. The S127L mutant was included in the later transfections as an internal control of a receptor with decreased signaling properties.

Cellular localization of the mutant and wt MC4-receptors was examined by fluorescence microscopy. All mutants and the wt receptor could be detected on the cell membrane by fluorescence microscopy (Figure 8 and study II, Figure 3A). Quantification of mutant receptors T112M, S127L and I226T on the cell surface was also performed with ELISA (study II, Figure 3B). It was shown that same amounts of mutant and wt receptors were transported to the cell membrane.

2.2 Functional studies of the *MC3R* promoter

In study I, the variants that were located in UTRs were analyzed *in silico* by use of the MatInspector program (Quandt et al. 1995). According to MatInspector, the -239A>G variant was located in a consensus GATA transcription factor binding site (aaacaaGATAaaaact), comprising nucleotides -244 to -228 of the *MC3R* gene (-239A>G major allele is underlined in the consensus sequence).

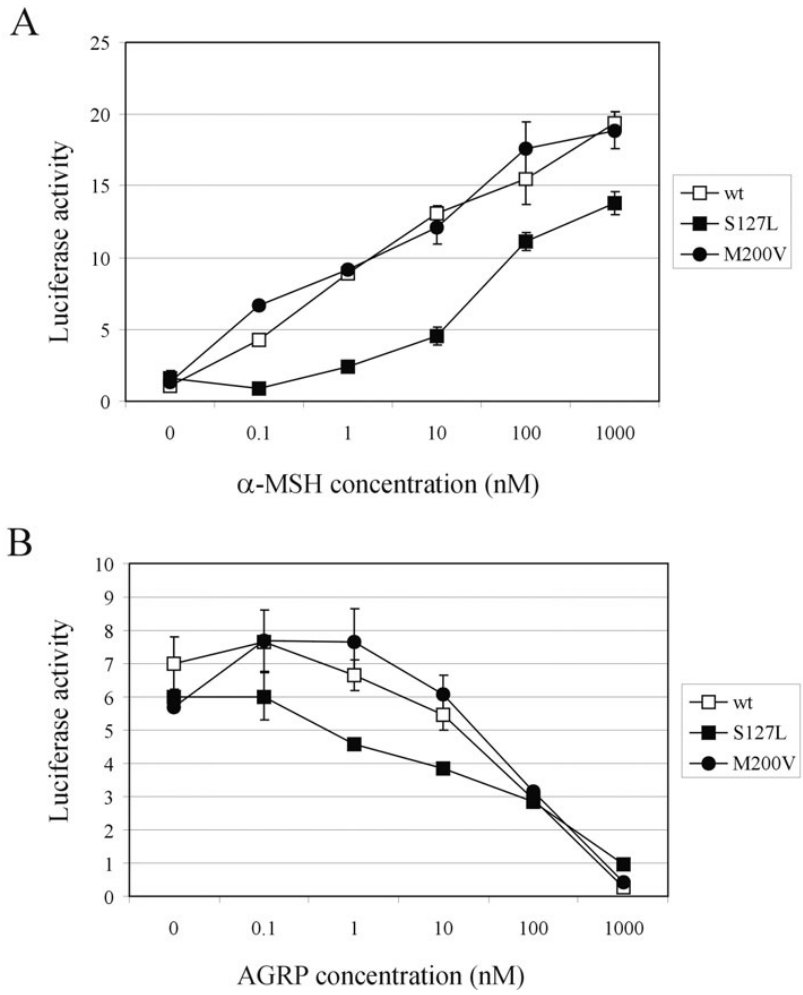

Figure 7. Luciferase reporter assay of receptor signaling. A) Responses of wt and mutant (S127L and M200V) MC4R to different doses of α-MSH. B) Responses of wt and mutant MC4R to different doses of AGRP, in a constant α-MSH concentration. Relative activity of the maximal receptor activity is shown.

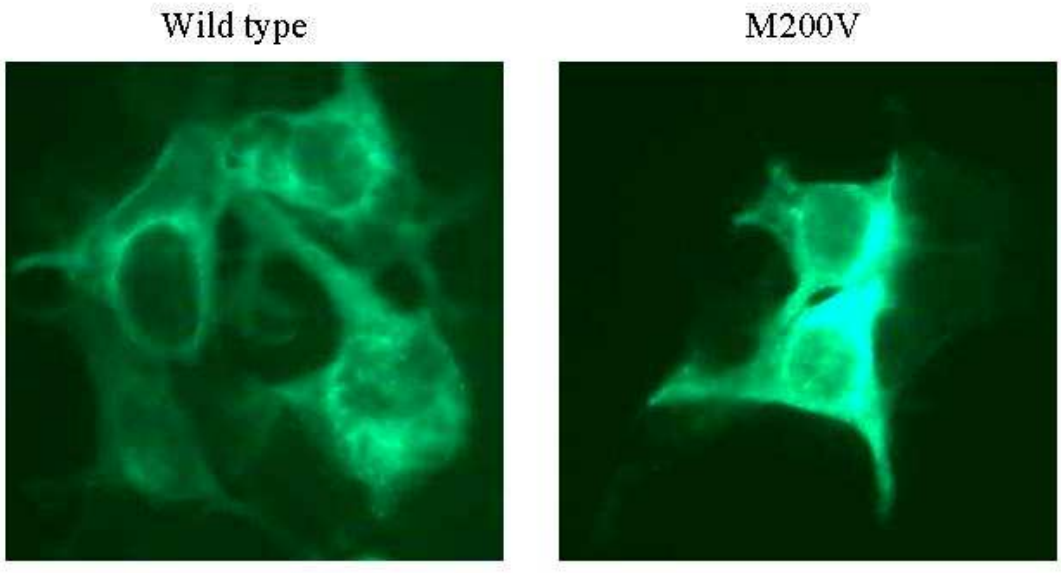

Figure 8. Wt MC4R and the M200V mutant were transiently transfected to 293H cells. The receptors could be detected on the cell surface by fluorescence microscopy.

The ability of the -239A>G site in the *MC3R* promoter to bind GATA proteins was assessed by use of EMSA. In the EMSA analyses, nuclear extracts containing GATA-4 and GATA-6 proteins yielded a strong retarded band A with the wt probe MC3R (study I, Figure 1, lane 1), whereas the MC3Rmut oligonucleotide revealed only a weak protein-DNA complex (study I, Figure 1, lanes 9 and 10). In competition assays, using an excess of unlabelled MC3R or SF-1 oligonucleotide (known to harbor a consensus GATA binding site), band A was clearly attenuated (study I, Figure 1, lanes 2, 3 and 5), indicating specific binding. Competition with unlabelled MC3Rmut oligonucleotide did not change the formation of band A (study I, Figure 1, lane 4). In supershift experiments, GATA-4 antibody abolished band A indicating that GATA-4 is responsible for binding to the MC3R oligonucleotide. In contrast, GATA-3 and GATA-6 antibodies did not change the protein binding pattern (study I, Figure 1, lanes 6, 7 and 8). Binding of GATA-4 was stronger to the wt oligonucleotide MC3R than to the MC3Rmut oligonucleotide (containing the minor allele of -239A>G).

2.3 Functional studies of the *MC4R* promoter

The -439delGC site in the *MC4R* promoter was analysed *in silico* by MatInspector and TFSearch software to search for important transcription factor binding sites that might be altered by the deletion. Both programs predicted the deletion to be located immediately 3' from a potential NHLH transcription-factor-binding E-box element (study III, Figure 1). According to MatInspector and TFSearch software, the NHLH-binding site is destroyed by the -439delGC deletion.

Binding of NHLH2 to the *MC4R* promoter region containing the -439delGC mutation was demonstrated by EMSA (study III, Figure 4). In the EMSA analyses, nuclear extracts containing NHLH2 were shown to bind to both wt (MC4R-wt) and mutant MC4R (MC4R-del) oligonucleotides, as well as to the μE2 control oligonucleotide harbouring an NHLH-binding E-box site (Uittenbogaard et al. 1999) (study III, Figure 4).

The activities of the Mc4rwt or Mc4rdelGC promoters were compared by luciferase reporter gene assays in HeLa and COS-1 cells. Reporter gene assays were also performed with a mouse pituitary gonadotrope cell line, LβT2. The Mc4rdelGC construct had similar luciferase activity as the Mc4rwt in all cells tested (data not shown). The activities of the Mc4r promoter constructs were several fold higher when compared to the empty pGL3-basic vector.

3 SNPS AND SNP HAPLOTYPES IN OBESITY CANDIDATE GENES *MC2R, MC3R, MC4R, MC5R, POMC* AND *ENPP1*

In study IV, a series of SNPs in the *MC2R-MC5R, POMC* and *ENPP1* genes were studied for association with obesity. Twenty-five SNPs, including 2-7 SNPs in each gene were genotyped in 246 morbidly obese Finns (BMI ≥ 40 kg/m^2) and 481 lean subjects (BMI 20-25 kg/m^2). The SNPs and SNP haplotypes were tested for association with obesity and type 2 diabetes.

Allele frequencies differed significantly between obese and lean subjects for two SNPs in the *ENPP1* gene when tested by a chi-squared test; rs1800949 (minor allele frequencies 0.22 and 0.16 in cases vs. controls, P = 0.006) and rs943003 (minor allele frequencies 0.48 and 0.39 in cases vs. controls, P = 0.0009). When corrected for by false discovery rate, the association remained significant for both SNPs rs1800949 and rs943003 (P-values 0.004 and

0.008, respectively). These SNPs are part of a haplotype (rs1800949 C-rs943003 A), which was observed more frequently in lean compared to obese subjects (P = 0.0007).

Suggestive associations were detected between the SNPs rs1541276 in the *MC5R* gene, rs1926065 in the *MC3R* gene and obesity (P = 0.04 and P = 0.03, respectively), and between SNPs rs2236700 in the *MC5R* gene, rs2118404 in the *POMC* gene, rs943003 in the *ENPP1* gene and type 2 diabetes (P = 0.03, P = 0.02 and P = 0.02, respectively). These associations with type 2 diabetes were detected in the presence of morbid obesity. None of these suggestive associations remained significant after correction for multiple testing.

ENPP1 SNPs rs1800949 and rs943003, located 5' to the transcription start point and in intron 1, were initially analyzed by TFSearch and TESS software. Both of these programs predicted the rs1800949 SNP to be located in an Upstream stimulatory factor 1 (USF1)-binding site and this site to be destroyed by the minor allele of the SNP. TFSearch predicted the SNP rs943003 to be located in a Lymphoid transcription factor 1 (LyF-1) binding site, whereas TESS predicted the binding sites for the following transcription factors to be located at this position: E1A-associated protein p300, Olfactory neuron-specific transcription factor-1 (Olf-1) and Drosophila hunchback (Hb). Further analyses were performed with ConSite and Con Real programs permitting analysis of conserved regulatory elements that are possibly affected by SNPs. Using these programs, no conserved regulatory elements at the location of the SNPs rs1800949 or rs943003 were detected when aligning human *ENPP1* sequences to the corresponding sequences from the species *Mus musculus*, *Rattus norvegicus*, *Pan troglodytes* or *Canis familiaris*.

DISCUSSION

1 GENETIC VARIATION IN THE *MC4R* GENE

1.1 Spectrum of *MC4R* mutations and polymorphisms

Mutations in the *MC4R* gene constitute the most common monogenic cause of human morbid obesity, as demonstrated by several studies finding significant association between severe early-onset obesity and *MC4R* mutations (Gu et al. 1999; Hinney et al. 1999; Sina et al. 1999; Farooqi et al. 2000; Vaisse et al. 2000; Dubern et al. 2001; Mergen et al. 2001; Hebebrand et al. 2002; Jacobson et al. 2002; Miraglia Del Giudice et al. 2002; Biebermann et al. 2003; Donohoue et al. 2003; Farooqi et al. 2003; Hinney et al. 2003; Lubrano-Berthelier et al. 2003b; Marti et al. 2003; Santini et al. 2004; Buono et al. 2005; Larsen et al. 2005; Hinney et al. 2006; Lubrano-Berthelier et al. 2006; Rong et al. 2006; Wang et al. 2006; Hainerová et al. 2007; Ochoa et al. 2007). In the present study, six pathogenic mutations in the *MC4R* gene were identified among Finnish children with severe early-onset obesity. Four of these mutations were located in the coding region of *MC4R* (308delT, two patients with S127L and P299H). A deletion -439delGC in the promoter region of *MC4R* was identified in two children. Additionally, a large number of polymorphisms were detected in the coding region (V103I, T112M, M200V, I226T, I251L) and in non-coding regions (-1042C>T, -1005C>T, -896C>T, -719G>A, -483C>T, -178A>C in the 5'UTR and 1059C>T in the 3'UTR) of the *MC4R* gene in all cohorts studied.

In the present study, the prevalence of apparently pathogenic *MC4R* mutations among Finnish children with severe early-onset obesity was 3.0%. This figure is close to the frequency of 2.98%, which represents the combined number of *MC4R* mutations identified in early-onset obesity from different study samples (Lubrano-Berthelier et al. 2006). Some studies suggest a quite high population prevalence (2.35-2.5%) of *MC4R* mutations among adult morbidly obese patients (Larsen et al. 2005; Lubrano-Berthelier et al. 2006). In line with several other studies finding much lower frequencies of *MC4R* mutations among adult obese patients (Jacobson et al. 2002; Branson et al. 2003; Santini et al. 2004; Hinney et al. 2006), no pathogenic *MC4R* mutations were found among Finnish morbidly obese adult patients. It is not known whether this implies amelioration of the phenotype upon aging in mutation carriers, or reflects an adverse effect of the *MC4R* mutations on survival.

1.2 Genetic variants in the *MC4R* promoter region

The *MC4R* gene promoter was not characterized until 2003 and the initial screening of 431 obese subjects revealed no mutations (Lubrano-Berthelier et al. 2003a). In Finnish morbidly obese children and adults several genetic variants were detected in non-coding regions of the *MC4R* gene. Especially interesting is the -439delGC deletion, which was detected in two children with severe early-onset obesity. The mutation could not be identified in 454 lean controls.

The deletion is situated in the minimal promoter region of the *MC4R* gene (Lubrano-Berthelier et al. 2003a), at a potential bHLH-binding E-box element, 12 nucleotides upstream of the major transcription start point. Interestingly, as predicted by MatInspector and TFSearch software, this E-box harbors a consensus-binding site for the NHLH transcription

factors. The transcription factor NHLH2 was recently implicated in the regulation of body weight in rodents, as *Nhlh2* deficient mice demonstrate an obesity phenotype similar to that seen in *Mc4r* deficient mice (Coyle et al. 2002). In addition, Jing et al. (2004) demonstrated that in mice, *Nhlh2* is expressed in all hypothalamic regions involved in body weight regulation, including *Mc4r* and *Lepr* expressing neurons. Given the overlapping expression patterns of *Nhlh2* and *Mc4r* in the hypothalamus and the fact that the *MC4R* promoter contains bHLH-binding sites (Lubrano-Berthelier et al. 2003a), it seems plausible that NHLH2 could influence *MC4R* expression. It was therefore hypothesized, that the -439delGC site identified in the present study could serve as an NHLH2 binding site, and that the two-nucleotide deletion might affect binding of this transcription factor. This in turn could result in altered promoter activity and gene expression.

By use of gel shifts assays it was demonstrated that the transcription factor NHLH2 indeed binds to the consensus E-box sequence at the -439delGC site *in vitro*. However, reporter gene assays performed in three different cell lines, including a mouse pituitary cell line, revealed no significant differences in mutant compared to wt *MC4R* promoter activities. This might have several different explanations. First, the influence of the two-nucleotide deletion on the function of the *MC4R* promoter may be too subtle to be monitored by transient transfection experiments and yet have phenotypic effects *in vivo*. Second, it is possible that the *MC4R* promoter fragment inserted in the reporter constructs was too short to adequately reflect differences in promoter activities. Third, the *in vitro* cell models may lack important cofactors that exist *in vivo*. Fourth, it is also possible that NHLH2 is not the transcription factor that binds to this specific region *in vivo*, but some other E-box binding bHLH factor may be more important for the regulation of the *MC4R* gene at this site.

1.3 Functional properties of MC4Rs and classification of mutations

Mutations in the *MC4R* include in-frame deletions, frameshift, nonsense and missense mutations scattered throughout the coding sequence of the gene, impairing signaling through the receptor by three major mechanisms, i.e. intracellular retention of the receptor leading to reduced cell surface expression, mutations resulting in reduced receptor activity in response to agonists and decrease in the constitutive activity of the receptor (Farooqi et al. 2003; Lubrano-Berthelier et al. 2003b). *In vitro* functional studies of natural mutations have revealed specific functional defects behind mutant MC4Rs (Gu et al. 1999; Ho and MacKenzie 1999; Farooqi et al. 2000; Vaisse et al. 2000; Kobayashi et al. 2002; Biebermann et al. 2003; Donohoue et al. 2003; Farooqi et al. 2003; Hinney et al. 2003; Lubrano-Berthelier et al. 2003b; Nijenhuis et al. 2003; Tao and Segaloff 2003; Yeo et al. 2003; Lubrano-Berthelier et al. 2004; Santini et al. 2004; Larsen et al. 2005; Tao 2005; Hinney et al. 2006; Lubrano-Berthelier et al. 2006). Dominant negative effects of the mutations have been excluded, suggesting haploinsufficiency as the major mechanism behind the obesity phenotype (Yeo et al. 2003). In a recent study, it was demonstrated that over 90% of inactivating *MC4R* mutations were located at evolutionary highly conserved amino acid locations (Stäubert et al. 2007).

In this study functional properties of four MC4R variant receptors were investigated (T112M, S127L, M200V and I226T), based on the facts that in our material the T112M, S127L and M200V variants were present in obese individuals only and no comprehensive information on the functional properties of these receptors were available. As there is growing evidence that α-MSH may not be the most important MC4R agonist in the brain (Mountjoy et al. 1999; Millington et al. 2001; Harrold et al. 2003; Biebermann et al. 2006; Lee et al. 2006), we took a novel approach and studied the signaling properties of the variant receptors, in addition to stimulation with α-MSH, also with β-MSH and γ_1-MSH. These studies confirmed

the pathogenic role of the S127L mutation, showing decreased responses to all the agonists tested. The data indicate that obesity in S127L carriers may, in addition to decreased signaling in response to α-MSH, also be due to decreased signaling in response to β-MSH. In contrast, γ_1-MSH is a poor ligand for MC4R *in vitro* and it seems unlikely that the observed decreased response to very high doses of γ_1-MSH plays a significant role in the pathogenesis of obesity in S127L carriers. We also tested the hypothesis that mutant MC4Rs could respond differently, in a mutation dependent manner, to these peptides. The results do not, however, support such a hypothesis. In contrast to the S127L mutation, the responses of the T112M, M200V and I226T variants did not differ from that of wt receptor. The variant receptors were also tested for inhibition by AGRP, but the mutations did not affect AGRP mediated inhibition of MC4R *in vitro*. Cell membrane localization of the variants T112M, S127L, M200V and I226T did not differ from that of wt. This confirmed that the impaired function of S127L is the result of a signaling defect and not a defect of transport to the cell membrane.

While study II was in progress, Lubrano-Berthelier et al. (2003b) reported, in line with the present data, that the S127L mutation was characterized by decreased activation of the receptor by α-MSH and not by impaired cell surface expression. The S127L mutation was also detected in compound heterozygous form in two subjects with severe, early-onset obesity (Hinney et al. 2003). In contrast to study II and the study by Lubrano-Berthelier et al., Hinney and co-workers concluded that the S127L mutation led to increased constitutive activity of the receptor, as they found a slight, twofold increase in basal S127L activity compared to the wt receptor (Hinney et al. 2003). Hinney et al. used a 1-hour cAMP assay as opposed to the 5-hour transcriptional assay used in the present study, which might provide one explanation for the differing results. However, importantly, in the study by Hinney et al. there was a major shift to the right of the dose response curve, compatible with severely impaired function of the S127L receptor (Hinney et al. 2003).

As the *MC4R* 308delT deletion is predicted to result in a grossly truncated receptor of only 107 amino acids, we did not perform functional studies on this mutant receptor. It has been previously shown that especially some C-terminal residues, which are totally absent from the mutant 308delT receptor, are important in MC4R cell surface targeting (VanLeeuwen et al. 2003).

Polymorphisms of the *MC4R* gene may modulate the obesity phenotype and some of the gene variants have been implicated in common polygenic obesity (Rankinen et al. 2006). For example, the V103I variant was shown to be negatively associated with obesity in three recent meta-analyses (Geller et al. 2004; Heid et al. 2005; Young et al. 2007). Although functional studies did not reveal differences between wt and variant receptors, the polymorphism was suggested to cause moderate gain of function of MC4R (Geller et al. 2004). It was also demonstrated that carriers of the variant allele I103 had significantly lower BMI compared to those carrying the wt allele V103 (Heid et al. 2005; Young et al. 2007). In addition, in a recent meta-analysis the I251L variant was shown to be negatively associated with obesity (Stutzmann et al. 2007). In the present study, no significant differences in allele frequencies of the V103I and I251L variants could be demonstrated between the groups due to small sample sizes. However, the 103I and 251L alleles seem to more frequently present among the background population if compared to the obese cohorts. In line with the present data, the *MC4R* variant M200V was proposed to be a polymorphism with similar signaling properties as the wt receptor (Tao 2005; Hinney et al. 2006). The variant receptors V103I, T112M, and I251L have also been shown to have normal signaling properties (Gu et al. 1999; Ho and MacKenzie 1999; Vaisse et al. 2000; Lubrano-Berthelier et al. 2003b; Tao and Segaloff 2005; Hinney et al. 2006). However, this was challenged by a recent study where Hinney et al. found reduced cell surface expression and ligand binding by the T112M receptor (Hinney et al. 2006).

Recently, two schemes for the classification of different types of *MC4R* mutations were proposed (Tao and Segaloff 2003; Govaerts et al. 2005). The classification of mutations is based on the observation of a positive relationship between the presence of a functional defect and both the onset of obesity and severity of the obesity phenotype (Govaerts et al. 2005). The genetic variants of the *MC4R* gene detected in Finnish obese patients were classified according to the scheme proposed by Tao and Segaloff (2003) (Table 10). According to that scheme the mutations can be divided into the five categories. Class I mutations are null mutations producing no receptor due to defective synthesis and possibly accelerated degradation of the defective protein. Class II mutations are intracellularly trapped mutants, which are not transported to the cell membrane. Mutations belonging to class III are defective in binding affinity and therefore have impaired agonist stimulated signaling properties. Class IV mutants have normal ligand binding ability, but the signaling properties are impaired. Variants with apparently normal function belong to class V.

Table 10. Classification of Finnish *MC4R* mutations according to the scheme proposed by Tao and Segaloff (2003).

Mutation class	*MC4R* mutation	Functional properties	Obesity phenotype
I	308delT	Null mutation	Severe early-onset obesity
II	P299H	Intracellular retention	Severe early-onset obesity
IV	S127L	Signaling defective	Severe early-onset obesity
V	V103I	Normal	Negatively associated with obesity, protective
V	T112M	Normal	Not associated with obesity
V	M200V	Normal	Not associated with obesity
V	I226T	Normal	Not associated with obesity
V	I251L	Normal	Not associated with obesity
-	-439delGC	Normal promoter activity	Severe early-onset obesity

1.4 Genotype-phenotype correlations of *MC4R* mutations

In contrast to several other monogenic obesity syndromes, *MC4R* deficiency is not easily detected even by careful clinical characterization of the patient, because of the lack of additional clinical characteristics or biochemical abnormalities besides obesity (Mutch and Clement 2006b).

The deletion 308delT (Class I) was identified in a 13.3-year-old girl, who began her abnormal weight gain before the age of one year and whose current BMI is 45.9 kg/m^2. She was diagnosed with type 2 diabetes at the age of 13. No clear segregation pattern of the obesity phenotype with the mutation could be detected in the family (Figure 4, page 36). The index patient's mother was shown to be a carrier of the 308delT mutation. She is currently overweight and was severely obese in childhood. Several other members of the family are overweight or obese, and in addition, several members of the family have type 2 diabetes.

The missense mutation P299H (Class II) was identified in a 9.3-year-old boy, whose abnormal weight gain began in the second year of life. In line with previous phenotypic data from other *MC4R* mutation carriers (Farooqi et al. 2003), the patient had severe fasting hyperinsulinemia. No clear segregation of the mutation with the obesity phenotype could be detected in the family, as one lean adult member of the family was shown to be a carrier of the mutation (Figure 4, page 36).

The S127L mutation (Class IV) was detected in two patients, a 14.8-year-old boy (index A) with a current BMI of 36.7 kg/m^2 and a 12.5-year-old girl (index B) with a BMI of 33.6 kg/m^2. The phenotype of index patient A was observed to be somewhat more severe than the phenotype of index patient B. Index patient A's abnormal weight gain began in the second

year of life, whereas index patient B started abnormally gaining weight at the age of five years. In addition, index A had severe fasting hyperinsulinemia and presented with acanthosis nigricans, which together point towards marked insulin resistance (Nguyen et al. 2001; Hirschler et al. 2002). In family A, a segregation pattern compatible with dominant inheritance of this mutation can be seen, as both mutation carriers are obese and the other family members are lean (Figure 4, page 36). In contrast, in family B one lean sibling of the index patient was shown to be a carrier of the mutation (Figure 4, page 36). The other mutation carriers in family B are severely obese. Both S127L index patients were shown to be carriers of the possibly protective gene variant V103I. It is unclear to what extent the effect of the S127L mutation can be overcome by the positive effects of the V103I polymorphism. This might to some extent explain the variable obesity phenotypes observed in the families.

The index patients carrying the *MC4R* promoter deletion -439delGC, currently aged 13.8 and 10.1 years, are severely obese with BMIs of 33.5 kg/m^2 and 31.2 kg/m^2, respectively. In both patients the abnormal weight gain began at the age of four years. Family studies and weight chart data revealed that the -439delGC deletion-carrying siblings of the index patients were characterized by early-onset obesity, as opposed to non-carrier siblings, whose weight charts demonstrated normal weight development. Other family members carrying the mutation have been lean throughout adulthood. These mutation carriers had a history of being overweight in childhood but this was not as pronounced as in the younger generation.

In families with *MC4R* mutations, the transmission mode seems to be autosomal dominant, although the penetrance of the disease can be incomplete. It has been demonstrated that the carriers of homozygous mutations develop a more severe phenotype than heterozygous patients (Farooqi et al. 2000; Lubrano-Berthelier et al. 2004; Dubern et al. 2007). The observation that severely obese members carrying wt alleles and lean carriers of mutations can be found in families with *MC4R* mutations further complicates the assessment of the genetic effects of these mutations. It is clear that environmental factors may have a large impact on the development of morbid obesity. According to the “thrifty gene” theory, the current environment might be more obesity-promoting for the children carrying *MC4R* mutations (Hinney et al. 2006), compared with the environment in which the older generations grew up, thus having a large impact on the phenotype of young *MC4R* mutation carriers. In line with this, Irani et al. (2005) demonstrated that hyperphagia is reduced and the obesity phenotype of *Mc4r* deficient mice can be overcome if exercise is introduced at an early stage. Furthermore, *Mc4r* deficient mice are not hyperphagic when fed a low-fat diet, but hyperphagia is observed when they are fed diets containing an increased amount of fat (Butler and Cone 2003). One interesting feature of *MC4R* mutations is that the phenotype of mutation carriers tends to ameliorate with time. Adult mutation carriers report less intense hunger feelings (Farooqi and O'Rahilly 2006).

It has previously been reported that *MC4R* mutation carriers are hyperinsulinemic in childhood (Farooqi et al. 2003). This can also be noticed in several of the Finnish children carrying *MC4R* mutations. The susceptibility for hyperinsulinemia is interesting in the light of our finding of a patient with the *MC4R* 308delT deletion and early-onset type 2 diabetes. Severe early-onset obesity, together with a familial predisposition for type 2 diabetes, might explain the early-onset of the disease in this patient.

It has been suggested that *MC4R* mutation carriers have accelerated linear growth in early childhood and an increase in lean mass and bone mineral density (Farooqi et al. 2003). In general, slight acceleration in growth can be seen in Finnish patients carrying *MC4R* mutations (standard deviation score, SDS of height varies from +0.2 to +2.4). For example, S127L index patient A was at the age of 12.8 years moderately advanced in skeletal development with a bone age of 14.0 years. Similarly, the -439delGC index patient B was at

the age of 10 years advanced in skeletal development with a bone age of 12.5. Thus they were tall for their age as obese children in general are (Russell et al. 2001; Cameron et al. 2003). The accelerated growth could be a consequence of early hyperinsulinemia seen in the patients (Farooqi et al. 2003) and the increase in bone density may be explained by a decrease in bone resorption (Elefteriou et al. 2005; Ahn et al. 2006).

An association between binge-eating disorder, i.e. compulsive overeating, and *MC4R* mutations was suggested by Branson and co-workers in 2003. This theory has been lively debated and so far has not been confirmed by others (Hebebrand et al. 2004; Tao and Segaloff 2005; Tortorella et al. 2005; Lubrano-Berthelier et al. 2006).

Based on these genotype-phenotype correlations of *MC4R* mutation carriers, the severity of the receptor defect appears to be associated with the time of onset and the degree of obesity (Table 11). Classification of *MC4R* mutations may provide a useful tool for predicting the outcome of the disease (Govaerts et al. 2005).

Table 11. Genotype-phenotype correlations of *MC4R* mutations in Finnish patients: classification of mutations and severity of the obesity phenotype.

Mutation class	MC4R mutation	Gender	Age at onset of abnormal weight gain (years)	Age (years)	BMI (kg/m^2)
I	308delT	Female	<1	13.3	45.9
II	P299H	Male	2	9.3	39.2
IV	S127L	Male	2	14.8	36.7
IV	S127L	Female	5	12.5	33.6
-	-439delGC	Female	4	13.8	33.5
-	-439delGC	Male	4	10.1	31.2

2 GENETIC VARIATION IN THE *MC3R*, *POMC* AND *NHLH2* GENES

Studies in animal models, human genome scans and case-control association studies all favor the role of *MC3R* and *POM*C in obesity. As the role of *NHLH2* has not been studied before in human morbid obesity, the evidence of the possible importance of this gene in obesity stems from animal studies. Table 12 summarizes the genetic variations identified in *MC3R, POMC* and *NHLH2* genes in the present study.

Allele frequencies of genetic variants (-239A>G, T6L and V81I) in the *MC3R* gene were determined in the cohorts of morbidly obese adults, children with early-onset obesity and a background population sample of healthy blood donors. The variants were common in all groups studied and therefore don't explain morbid obesity. Among morbidly obese adults, the minor alleles of T6L and V81I variants were associated with higher leptin levels and higher insulin-glucose ratios. This was in line with the results by Hani and co-workers (2001), who found marginally increased insulin-glucose ratios during an OGTT in normoglycemic subjects carrying the T6L and V81I variant alleles.

The *MC3R* -239A>G variant is located in a consensus GATA transcription factor binding site, as predicted by the MatInspector program. By use of gel shift assays we were able to demonstrate that, of the various GATA proteins, GATA-4 is responsible for specific binding to the *MC3R* -239A>G site. In addition, it was demonstrated that the binding of GATA-4 is stronger to the wt allele than to the variant minor allele of -239A>G.

Table 12. Genetic variations detected in the *MC3R, POMC* and *NHLH2* genes in Finns.

Gene	Genetic variation	Type of variation	Location within the gene	Reference
MC3R	-939G>C	nucleotide substitution	5'UTR	I
	-911G>A	nucleotide substitution	5'UTR	I
	-803T>C	nucleotide substitution	5'UTR	I
	-373G>T	nucleotide substitution	5'UTR	I
	-335G>A	nucleotide substitution	5'UTR	unpublished
	-239A>G	nucleotide substitution	5'UTR	I, Li et al. 2000
	-135insCT	insertion	5'UTR	unpublished
	T6L	missense	exon 1	I, Li et al. 2000 Hani et al. 2001
	V81I	missense	exon 1	I, Li et al. 2000 Hani et al. 2001
	327G>A (P109P)	silent nucleotide substitution	exon 1	unpublished
	354G>C (A118A)	silent nucleotide substitution	exon 1	unpublished
	L249V	missense	exon 1	unpublished
	1091G>A	nucleotide substitution	3'UTR	unpublished
POMC	C7662T (S68S)	silent nucleotide substitution	exon 3, JP	Hinney et al. 1998
	73/74insSSG	insertion	exon 3, JP	Hinney et al. 1998
	C7726T (L90L)	silent nucleotide substitution	exon 3, JP	Hinney et al. 1998
	C7965T (A169A)	silent nucleotide substitution	exon 3, γ-LPH	Hinney et al. 1998
	E188G	missense	exon 3, γ-LPH	Hinney et al. 1998
	C8246T	silent nucleotide substitution	exon 3, 3'UTR	Echwald et al. 1999
	G8469C	silent nucleotide substitution	exon 3, 3'UTR	unpublished
NHLH2	96C>T (L32L)	silent nucleotide substitution	exon 1	III

JP, joining peptide; γ-LPH, γ-lipotrophic hormone

A potentially interesting missense mutation, L249V, in the *MC3R* gene was identified in one morbidly obese patient. The variant could not be detected in the background population (n=312). Further studies of the L249V variant are needed in order to assess the functional consequence and possible co-segregation of the mutation with obesity in family members. Until now, only three rare *MC3R* mutations (A70T, M134I and I183N) have been described to be associated with obesity (Lee et al. 2002; Rached et al. 2004; Tao and Segaloff 2004; Lee et al. 2007). The first obesity associated *MC3R* mutation described was I183N, resulting in loss of function of the receptor (Lee et al. 2002; Rached et al. 2004; Tao and Segaloff 2004). The *MC3R* I183N mutation was identified in an obese 13-year-old girl and her father. Functional analysis of this mutation revealed a defect in the agonists mediated activation of the receptor (Tao and Segaloff 2004). In the same study, the T6K and V81I variants were shown to have normal signaling properties. In a recent study by Lee et al. (2007), the I183N mutation and two additional mutations, A70T and M134I, were shown to result in impared signaling activity of the receptor. These *MC3R* mutations were suggested to contribute as a predisposing factor in childhood obesity (Lee et al. 2007).

Several known genetic variants were detected in the *POMC* gene (Hinney et al. 1998; Echwald et al. 1999; Miraglia del Giudice et al. 2001; Rosmond et al. 2002; Feng et al. 2003; Suviolahti et al. 2003b; Santoro et al. 2004; Chen et al. 2005). Two previously described variants (73/74insSSG and E188G) and a novel polymorphism (G8469C) were selected for

determination of allele frequencies. Allele frequencies of these variants did not differ between morbidly obese adults, children with severe early-onset obesity and control subjects from the background population.

In study III, no pathogenic mutations were found in the *NHLH2* gene. However, one silent nucleotide substitution 96C>T (L32L) was identified in two patients. Thus, mutations in the *NHLH2* gene have not been associated with severe early-onset obesity in humans, despite attempts to identify *NHLH2* human mutations (Brennan et al. 2006).

3 A RISK HAPLOTYPE FOR OBESITY IN THE *ENPP1* GENE

The *MC2R, MC3R, MC4R, MC5R, POMC* and *ENPP1* genes are all relevant candidates for obesity. We took the approach of genotyping SNPs distributed over the coding and non-coding regions of these genes and tested the possible association of these SNPs and SNP haplotypes with obesity and type 2 diabetes.

The common allele of an *ENPP1* haplotype, composed of the SNPs rs1800949 and rs943003, was observed more frequently in lean compared to obese subjects. The *ENPP1* variant K121Q has frequently been studied for association with insulin resistance, type 2 diabetes and obesity. In this study, no associations between the K121Q variant and obesity or type 2 diabetes were detected, but other variants of the *ENPP1* gene were associated with obesity.

These two *ENPP1* SNPs, rs1800949 and rs943003, do not represent functional missense variants affecting the amino acid sequence of the protein. SNP rs1800949 is located 5’ to the transcription start point and rs943003 is located in the first intron of the *ENPP1* gene. No conserved regulatory elements at the location of these SNPs were detected by use of computational *in silico* analysis. These SNPs may nevertheless serve as regulatory elements of the *ENPP1* gene. Alternatively, these sites could be in LD with functional elements of the *ENPP1* gene or be in LD with a yet unidentified functional polymorphism in another gene.

In conclusion, an *ENPP1* haplotype was found to be associated with morbid obesity in Finnish adults.

CONCLUDING REMARKS AND FUTURE PROSPECTS

During the last ten years there has been remarkable progress in the field of obesity research. The identification of rare monogenic defects in human genes, encoding proteins of the leptin-melanocortin signaling system, has confirmed that inherited forms of human obesity indeed exist. Mutations in the *MC4R* gene underlie 1-6% of morbid obesity worldwide, thus *MC4R* deficiency represents the most common monogenic defect causing human obesity reported so far (Vaisse et al. 2000; Farooqi and O'Rahilly 2006). Functional testing of mutant proteins has become a valuable research tool in the evaluation of the consequences of a genetic defect. In addition, knowledge about the importance of regulatory regions in gene expression is continuously increasing.

The main achievement of this thesis was the identification of *MC4R* mutations in Finnish patients. In total, six pathogenic mutations were identified, giving a prevalence of 3% for *MC4R* mutations in severe early-onset obesity. No obesity causing *MC4R* mutations were found among Finnish patients with morbid adult-onset obesity. The *in vitro* functional properties of these mutant receptors were elucidated and new insights in the function of the *MC4R* gene were gained through studies of the promoter region.

Several common genetic variants were identified in the *MC3R* and *POMC* genes. These polymorphisms do not explain morbid obesity, but the results indicate that some of these genetic variations might be modifying factors in obesity, resulting in subtle changes in obesity-related traits. Thus, the minor alleles of the common *MC3R* T6L and V81I variants were found to be associated with higher leptin levels and higher insulin-glucose ratios. Furthermore, a risk haplotype for obesity in the *ENPP1* gene was identified through an approach of genotyping SNPs distributed over several candidate genes. An *ENPP1* haplotype, composed of SNPs rs1800949 and rs943003, was shown to be associated with morbid obesity in adults. The *MC3R*, *POMC* and *ENPP1* genes represent examples of susceptibility genes in which genetic variants predisposing to obesity have been identified.

In conclusion, pathogenic mutations in the *MC4R* gene were shown to account for 3% of cases with severe early-onset obesity in Finland. The severity of the MC4-receptor defect appears to be associated with time of onset and the degree of obesity. Thus classification of MC4R mutations may provide a useful tool when predicting the outcome of the disease. In addition, several other genetic variants conferring suseptibility to obesity were detected in the *MC3R*, *MC4R*, *POMC* and *ENPP1* genes.

Although several genes involved in the regulation of energy balance and appetite have been identified, the genetic basis of common obesity remains partly unsolved. The next goal in the post-genome era is to further characterize the variation in the human genome and to identify the factors underlying common, polygenic forms of obesity. It is probable that a combination of several approaches, including genome-wide SNP scans, transcriptomics, proteomics, metabolomics, pharmacogenomics and nutrigenomics are needed to unravel the complex gene-gene and gene-environment interactions underlying obesity.

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